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Studies on the Biosynthesis of Lignin

Part II. Changes of the Contents of the Enzymes and Organic Acids concerning the Lignification in the Growth of "Yugao" Fruits (*Lagenaria leucantha* Rosby var. *clavata* Makino)

By Isamu Tachi and Akira Sato

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University

Received February 23, 1960

We have been further studying on the specific lignification in the gourd fruits as shown in the previous paper. During the process of lignification, the both activities of peroxidase and β-glucosidase were decreased, and so were shikimic acid, while some organic acids including quinic acid were detected by paper chromatography.

INTRODUCTION

It has been confirmed by B. D. Davis[1] et al. that aromatic compounds in microbes were derived from shikimic acid. This process has also been confirmed in higher plants by S. A. Brown[2] et al. who experimented with the isotopic compounds in the wheat, and the acid has already been found in many plants.

On the other hand, as shown in the results of K. Freudenberg's[3] and T. Higuchi's[4] works, it has been known that coniferylalcohol derived from coniferine was polymerized to lignin-like materials by the aid of the enzyme which mainly consisted of laccase.

S. M. Siegel[5] and T. Higuchi[6] et al. have supposed that peroxidase mainly contributed to the polymerization of phenylpropanes, because of the wider distribution of the enzyme in higher plants and of its ability to produce lignin-like materials in vitro.

Expecting that there were some strong enzymatic actions on the basis of the phenomena of specific violent lignification in the gourd fruits as shown in the previous paper[7], we tried to investigate some organic acids and enzymes concerning lignification by using the fruits which were taken off after a suitable period after flowering.

EXPERIMENTAL AND RESULTS

The sample fruits used for enzymatic experiments were grown in the farm of Kyoto Univ. from March till August in 1959, and were put to experiment in their shell parts (epicarp and part of 1 cm thickness below greenish mesocarp). The dates of their flowering were decided by determing their lignin contents by the method of TAPPI[7], as it was found in previous paper that the lignin contents of the fruits increased in direct proportion to the days after flowering. The results are shown in Table I. Furthermore, the same kinds of fruits grown at Hikone in Shiga Pref. were used in a part of the experiments. An example of them is shown by a picture in Fig. 1.

1. Preparation of Enzyme Solution

Each fifty grams of material was brayed by a mixer with 100 ml of phosfate buffer, and homogenates were centrifuged after filtration with cloth. The supernatant fluids were filled up to 200 ml. These were mainly used to determine peroxidase. (see Fig. 2a) On the

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[1] B. D. Davis, Adv. in Enzymology, Vol. 16, p. 287 (1955).
[2] S. A. Brown and A. C. Neish, *Nature*, **175**, 688 (1955).
[3] K. Freudenberg, *Chem. Ber.*, **85**, 641 (1952).
[4] T. Higuchi, *J. Biochem.*, **45**, 515 (1958).
[5] S. M. Siegel, *J. Ame. Chem. Soc.*, **78**, 1753 (1956).
[6] T. Higuchi and Y. Ito, *J. Biochem.*, **45**, 575 (1958).
[7] I. Tachi, A. Hayashi and A. Sato. This Bulletin, **21**, 279 (1957).

TABLE I. "YUGAO" FRUITS USED SAMPLE

Sample	Weight	Around	Height	Lignin*	Days after Flowering
A	0.55 kg	30 cm	12 cm	3.56%	15
B	3.70	64	20	4.55	16
C	1.80	53	13	7.34	20
D	8.15	97	19	8.35	22
E	3.35	60	24	11.92	28

* based on air dry matter; determined as Klason lignin.

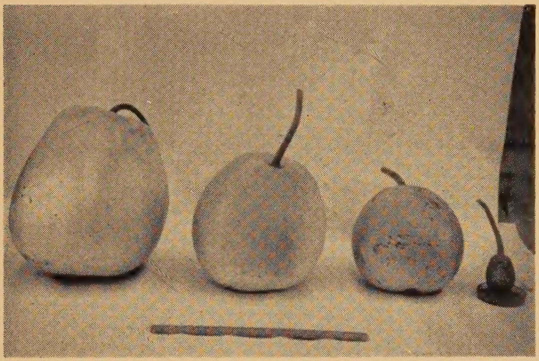

FIG. 1. The Picture of "Yugao" Fruits.

The one at the far right was taken off 5 days after flowering, weighing 86 g, and the rest 10 days (2.7 kg), 15 days (4.5 kg) and 23 days (6.6 kg), respectively from right to left. The front bar is 30 cm long.

other hand, 100 g of the same material was brayed with distilled water and the supernatant was saturated with ammonium sulfate. The precipitate obtained were dialysed against several changes of distilled water for 48 hours and used to determine β-glucosidase. Whole treatment was carried out in a room at 6°C. (see Fig. 2b)

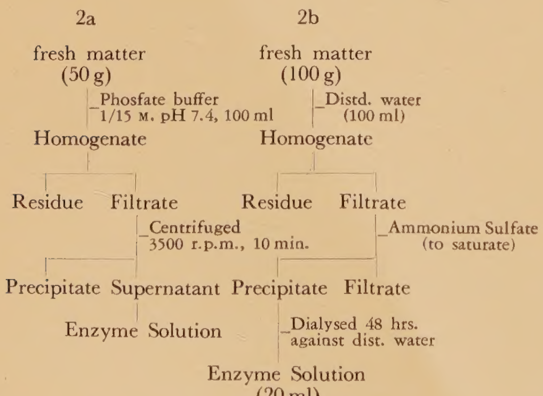

FIG. 2. Preparation of Enzyme Solution

2. Preparation of Sample Solution for Organic Acids

The method, as M. Hasegawa[8] et al. reported previously, was used for shikimic acid. Namely, each 300 g of material was brayed with 200 ml of methanol (80%) by a mixer and then was warmed on the bath at 60°C for one hour and filtered with cloth. The resulted residue was extracted with 200 ml of methanol as mentioned above. Both filtrates were mixed together and the mixture was condensed in vacuo to driness. The residues were dissolved in 40 ml of water and were poured into each column packed with 15 ml of Amberlite IRA–400 (carbonate form). Then, it was eluted with ammonium carbonate (1–N) and the elute was again dissolved in water and poured into a column of Amberlite IRC–50 (10 g, hydrogen form) to exchange cations. The solution was condensed in vacuo and was put to paper chromatography.

3. Peroxidase

The determination of peroxidase was carried out by the colorimetric method by K. Kondo and Y. Morita[9]. The activity was shown with quantities of tetraguajacol per one mg protein in solution. Folin's method[10] was used to determine protein. As shown in Fig. 3, the high activities in the crude enzyme were obtained at pH 4.8 and 5.4. Water extracts were also fractionated with adding ethanol up to 60～70% concentration. And the precipitate was dissolved in water and determined. The highest activity was obtained at pH 5.2. These facts are very interesting. In these respects, the experiment will be done in this summer in fruiting

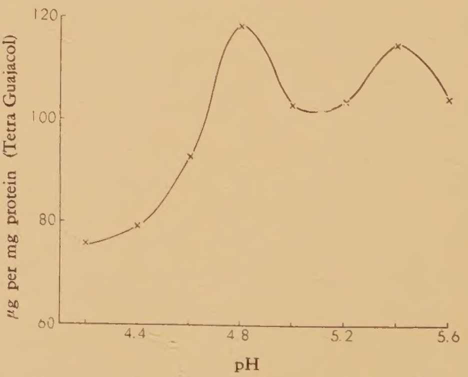

FIG. 3. Opt. pH of "Yugao" Peroxidase.

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[8] M. Hasegawa, S. Yoshida and T. Nakagawa, *Science*, **24**, 421 (1954).

[9] K. Kondo and Y. Morita, *Memoires of the Research Institute for Food Sci., Kyoto Univ.*, **4**, 12 (1951).

[10] O. Foline and V. Ciocalteu, *J. Biol. Chem.*, **73**, 629 (1927).

stage. Also, the results in addition of fluoride in the enzyme solution scarcely tended to decrease in the enzymatic activity, but cyanide showed great decrease as shown in Fig. 5. Comparison of the activity obtained in each sample after flowering was shown in Fig. 7.

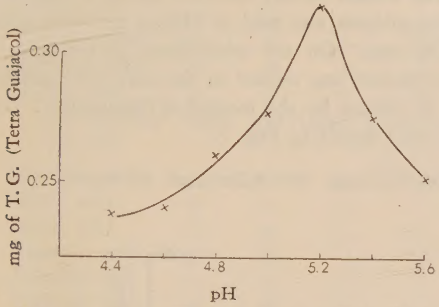

FIG. 4. Opt. pH of "Yugao" Peroxidase. (alcohol fractionated)

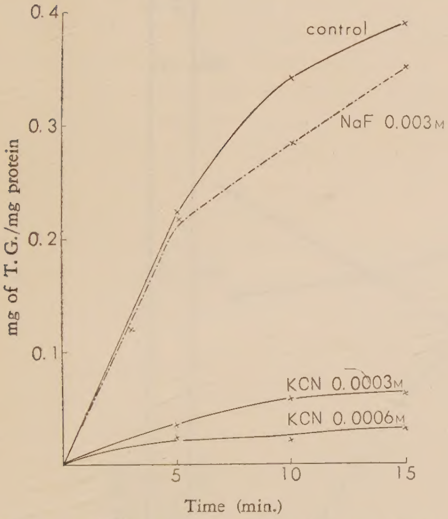

FIG. 5. Effect of added Fluoride and Cyanide on "Yugao" Peroxidase (pH 5.4).

4. β-glucosidase

It has already been known that β-glucosidase is useful when coniferylalcohol is derived from coniferine. So, we tried to detect the enzyme whether it was contained in the fruits or not, and to determine the glucosidase activity in them. The procedure was followed as described in T. Higuchi's paper[11], that is to say, the enzyme solution prepared in Fig. 2b was used, and the quantities of glucose was determined which was liberated from salicine at the enzymatic reaction of 30°C, 24 hours by the method of Schaffer-Somogyi[12]. From the results as shown in Fig. 6, the highest activity was obtained at pH 4.8 and the activity quickly decreased as the lignification proceeded as shown in Fig. 7.

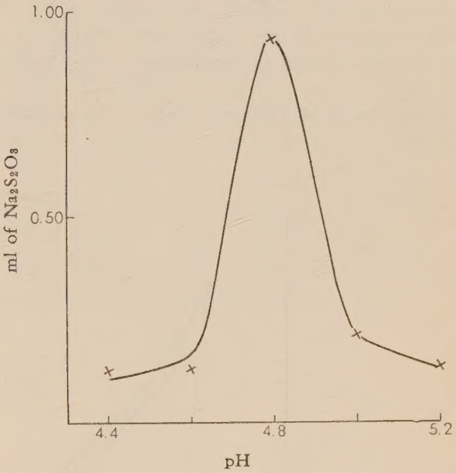

FIG. 6. Opt. pH of "Yugao" β-Glucosidase.

On the other hand, we tried to detect whether laccase was contained in the fruits or not, by using Warburg manometer and hydroquinone as substrate, but the results were negative. However, the results of quproine test which have been known to be useful in qualitative detection of the enzyme containing cuprous compounds were scarcely positive in some samples. We, therefore, supposed that the little enzyme existed in the fruits.

5. Paper Chromatography of Organic Acids

The solution of organic acids obtained above was subjected to paper chromatographic separation by using as solvent both ether-acetone-acetic acid-water (6 : 6 : 3 : 1 v/v)[13], and phenol-water-formic acid (85%) (3 : 1 w/w : 1%)[14]. The papers used were Toyo No. 50 and 51. As spray reagents, 0.05% B.P.B. alcohol solution and 0.5% $KMnO_4$ solution[15] were used for detection of general acids, while the reagent of Cartwright[16] and aniline reagent of Yoshida[17] were also used for identifying shikimic acid and quinic acid. Ascending method was used at room temperature. The spots were confirmed by comparison of authentic samples among themselves. From these results, as shown in Table II, it is evident that there were succinic, malic, tartaric, fumalic, quinic and shikimic acids in the fruits.

---

[11] T. Higuchi, I. Kawamura and H. Ishikawa, *J. Japanese Forestry Soc.*, **35**, 258 (1953).

[12] T. Souda and H. Egami, "Tatorui Kagaku" Kyoritsu Press, 1955, p. 54.

[13] F. W. Denison and E. F. Phares, *Anal. Chem.*, **24**, 1628 (1952).

[14] J. B. Stark, *Anal. Chem.*, **23**, 415 (1951).

[15] M. Hasegawa and H. Hirokawa, *J. Japanese Forestry Soc.*, **37**, 530 (1955).

[16] R. A. Cartwright and E. A. H. Roberts, *Chem. and Ind.*, **1955**, 230.

Furthermore, both shikimic and quinic acids were especially determined, for these were related to the process of aromatization in lignification. The quantitative determination of the former was carried out by the method of Yoshida et al. Namely, each 150 mg of the sample solution on the filter paper (Toyo No. 51, 40× 40 cm) was developed by the phenol solvent described with cold water up to 10 ml. Every 2 ml of this solution was treated further and the optical density of the resulting solution was read at 510 mμ by using a Beckman DU type. On the other hand, in the latter, the sample solution was treated in the same way and determined at 390 mμ by the method of Nagasawa[18]. These results were shown in Fig. 7.

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[17] S. Yoshida and M. Hasegawa, *Arch. Biochem. and Biophy.*, **70**, 377 (1957).

[18] M. Nagasawa, This Bulletin, **22**, 205 (1958).

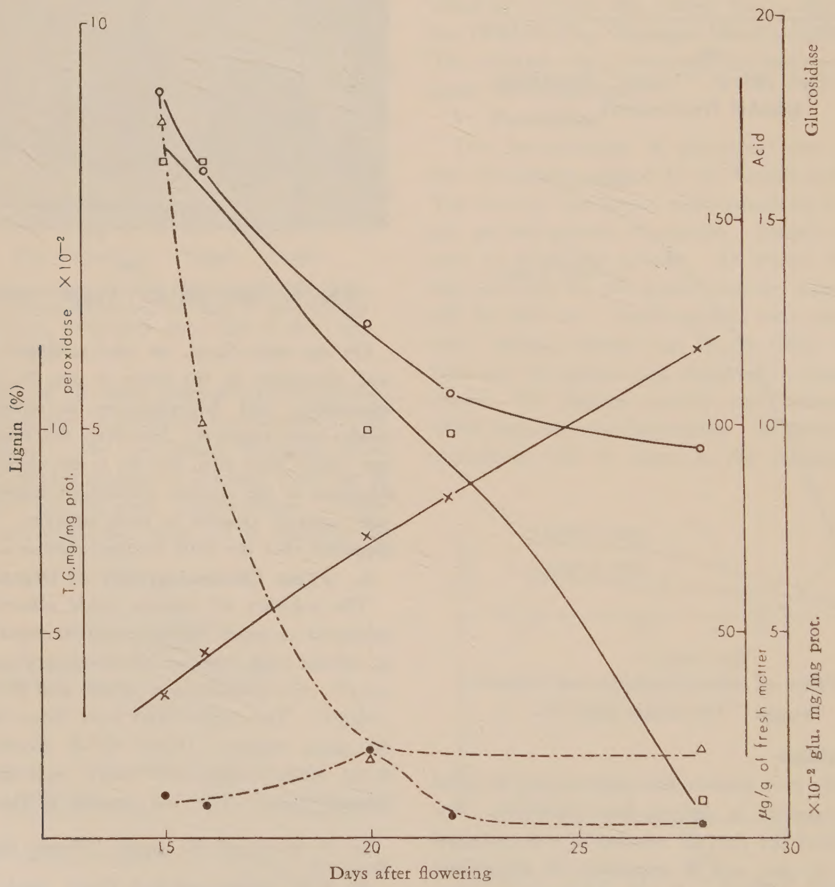

FIG. 7. Analysis of Some Components in "Yugao".

—×——×— lignin
—○——○— peroxidase
—□——□— glucosidase
—·△——△·— shikimic acid
—·●——●·— quinic acid

TABLE II. PAPER CHROMATOGRAPHY OF ORGANIC ACIDS IN "YUGAO"

Acid \ Sample	A′	B′	C′	D′	R_F
Succinic acid	−	±	−	±	0.84
Malic acid	+	+	++	+	0.64
Citric acid	+	±	±	±	0.50
Tartaric acid	++++	+++	++	++++	0.42
Shikimic acid	++	+++	++	++	0.34
Quinic acid	±	±	±	±	0.26

ether : acetone : acetic acid : water (6:6:3:1), Toyo 50

Acid \ Sample	A′	B′	C′	D′	R_F
Succinic acid	++	−	++	++	0.69
Malic acid	++	−	++	−	0.46
Citric acid	++	++	++	++	0.35
Tartaric acid	++++	++	++	++++	0.29
Fumalic acid	−	+	++	−	0.31
Shikimic acid	++	+++	++	++	0.66
Quinic acid	+	+	+	+	0.53

phenol : water : formic acid (3 : 1 : (1%)), Toyo 50

A′	22 days after flowering	very intense	+++, ++++
B′	15 〃 〃 〃	intense	++
C′	10 〃 〃 〃	positive	+
D′	5 〃 〃 〃	trace	±
		negative	−

SUMMARY

The higher activities were obtained at pH 4.8 and 5.4 in the crude enzyme of peroxidase and inhibited by potassium cyanide. β-glucosidase was present and its opt. pH was 4.8. Laccase was scarcely found in the fruits. Some organic acids were detected by paper chromatography; succinic, malic, citric, tartaric, quinic and shikimic acids. Especially, both enzymes and shikimic acid were shown to decrease with proceeding of lignification.

Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 638~643, 1960]

Vitamin and Amino Acid Requirements of *Pediococcus soyae* and *Pediococcus acidilactici* Kitahara's Strain

Part IX. Studies on the Activities of Bacteria in Soy Sauce Brewing

By Kenji SAKAGUCHI

Noda Institute for Scientific Research, Noda-shi, Chiba-ken

Received March 22, 1960

Nutritional requirements of three strains of *Ped. soyae*[1,2], one strain of *Ped. acidilactici* (Kitahara's strain) were determined from a complete synthetic medium. Upon inspection by the single omission method, *Ped. soyae* required glycine-betaine[3], uracil, riboflavine, pyridoxine or pyridoxal, pantothenic acid, nicotinic acid, leucovorin, biotin, glutamic acid, arginine, histidine, isoleucine, leucine, phenyl alanine, valine, tryptophane, methionine, cystine and serine with exception of the P-factor[4]. In addition to the above mentioned nutrients, a representative strain of the organism required xanthine, folic acid, thiamine, aspartic acid and threonine for the minimum synthetic medium. *Ped. acidilactici* Kitahara's strain required riboflavine, pyridoxine, pantothenic acid, nicotinic acid, biotin and all components of the basal medium consisting of amino acids except methionine, but it did not require leucovorin, glycine-betaine and organic bases. Nutritional requirements of *Ped. pentosaceus*, Kitahara's strain[5,6] was proved to be quite identical with *Ped. cerevisiae* Pederson[7~9] (=*Ped. pentosaceus* Mees[5,10]).

The genus *Pediococcus* was first attached to the family *Lactobacillaceae* in the 7th edition of Bergey's Manual[7] as the result of laborious works of Pederson[7~9] and others[10,11]. However, in this genus only two species are included, namely *Ped. cerevisiae* and *Ped. acidilactici*, and nothing concerning salt-tolerant and dextrorotatory lactic acid forming pediococci is described[1,2,5,12]. Moreover, Nakagawa and Kitahara[5] concluded that type cultures of the so-called *Ped. cerevisiae* classified by Pederson is not a true beer-sarcina known as *Pediococcus cerevisiae* Balcke, it should be included in *Ped. pentosaceus* Mees[5,12].

The nutritional requirements of *Ped. pentosaceus* were first studied by Sauberlich and Baumann[13] with a strain of this genus formerly known as *Leuconostoc citrovorum* ATCC 8081[8] and specific requirements for leucovorin (folinic acid) were elucidated. Thereafter, Jensen and Seeley have reported on the minimum synthetic medium for 34 strains of this species, and concluded that requirements of these strains for vitamins, bases and amino acids were quite uniform.

As for the thermotolerant pediococcus *Ped. acidilactici* Lindner, a strain of the species[5], *Leuconostoc mesenteroides* P-60 was thoroughly investigated for the purpose of microbiological

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[1] Kenji Sakaguchi, This Bulletin, **22**, 353 (1958); **23**, 22, 100, 438 (1959).

[2] K. Yamazato and H. Iizuka, *J. Agr. Chem. Soc. Japan*, **33**, 379, 383 (1959); *J. Gen. Appl. Microbiol.*, **5**, 58 (1959).

[3] Kenji Sakaguchi, This Bulletin, in press.

[4] Kenji Sakaguchi, *ibid.*, **23**, 443 (1959).

[5] A. Nakagawa and K. Kitahara, *J. Gen. Appl. Microbiol.*, **5**, 95 (1959).

[6] K. Kitahara, *J. Agr. Chem. Soc. Japan*, **16**, 697, 819 (1940).

[7] Bergey's Manual of Determinative Bacteriology, 7th ed., p. 529 (1957).

[8] C. S. Pederson, *Bact. Rev.*, **13**, 225 (1949).

[9] C. S. Pederson et al., *Wallerstein Lab. Comm.*, **17**, 7 (1954).

[10] E. A. Felton and C. F. Nieven, *J. Bact.*, **65**, 482 (1953).

[11] E. M. Jensen and H. W. Seeley, *ibid.*, **67**, 484 (1954).

[12] R. H. Mees, Onderzoekingen over de Biersarcina, Thesis, Delft (1934).

[13] H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, **176**, 165 (1948).

bioassay by Snell[14~16], Dunn[17] and Steele[18].

Ped. mevalovorus Kitahara et Nakagawa[19], a variant of *Ped. cerevisiae* Balcke[5], was reported to require mevalonic acid as a specific nutrient.

Recently, Nakagawa and Kitahara have offered a probable proposal on the general classification of the genus *Pediococcus*, and nutritional requirements of the member species were also studied. It is unfortunate that Difco's vitamin-free casamino acids were used as the amino acid source, as this acid hydrolysed milk casein is not actually vitamin-free, and contamination of unsaturated fatty acids[20], an unknown growth factor for *Ped. soyae* (P-factor)[4,21] and other substances as diketopyperazines or amines have been reported or suspected. On the whole it may be said that, Nakagawa and Kitahara's report, is, in most cases convincible as a general classification of the genus, but its nutritional description is imperfect especially in the case of *Ped. soyae*. Naturally, amino acid requirements of the genus were not referred in their report.

The present paper deals with the nutritional requirements of *Ped. soyae* inspected by the single omission method and by the minimum synthetic medium which comprises glycine-betaine[3] as a specific nutrient for the organism. Furthermore, the vitamin and amino acid requirements of *Ped. acidilactici* Kitahara's strain and *Ped. pentosaceus* Kitahara's strain are studied by the single omission method from the complete synthetic medium and the results obtained are offered for general studies of this genus and for subsequent studies of this series.

METHODS

Basal medium. The composition of the basal medium employed was identical with that listed in the previous report[3]. That is, the standard synthetic medium for lactic acid bacteria was supplemented with glycine-beatine hydrochloride, 30 mg/l, choline hydrochloride, 2 mg/l, inositol, 2 mg/l, and leucovorin (folinic acid) 10 μg/l, but pyridoxal was omitted here.

Cultures. Three strains of *Ped. soyae*, d 2, d 8 and dl 2 were selected from the author's isolates because of their heterogenity in sugar fermentabilities or optical rotation of produced lactic acid[1].

The thermotolerant strain *Ped. acidilactici* Kitahara's strain originally isolated from a corn mash by K. Kitahara[6] in 1940, and classified at that time as a strain of *Ped. lindneri* Henneberg. At the same time *Ped. pentosaceus* Kitahara's strain was isolated from another corn mash and was assigned its former name *Ped. hennebergi* Sollied. Recently, taxonomical identification of the two strains were investigated again by Nakagawa and Kitahara[5] and were given the name of new species.

Assay method. Procedures were identical with that described in the former reports[3,4]. After four days' subculture in the case of *Ped. soyae*, and after two day's in the case of *Ped. acidilactici* or *Ped. pentosaceus*, the cells were centrifuged, washed four times with 0.9% potassium chloride solution, and diluted to give a final optical density of approx. 0.07, at 660 mμ. Five-ml basal medium omitted with each one of the nutrients were dispensed in cotton-plugged test tubes, autoclaved for fifteen minutes at 120°C, cooled, incubated with one drop per tube of the cell suspension described above and incubated at 30°C. After incubation for an appropriate period, growth was estimated photometrically at 660 mμ, or the acid produced was titrated with N/20 sodium hydroxide solution. Quadruplicate tests for one sample were run as usual and growth was assayed periodically.

RESULTS AND DISCUSSION

I Vitamin and Base Requirements of *Ped. soyae*

Three strains of *Ped. soyae* d 2, d 8 and dl 2 which had different characters to each other in sugar fermentability and optical rotation of produced lactic acid were selected and inoculated into the media which were eliminated by one of each vitamin or base from the basal synthetic medium. Growth was estimated turbidimetrically after five and eight days' and acidmetrically after eight days' culture. The results shown in Table I indicate that all three strains essentially require uracial, riboflavine, pantothenic acid, nicotinic acid, leucovorin, pyridoxine, while stimulatory growth effects are observed

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14) E. E. Snell, Adv. in protein Chemistry, Vol. 2, 85 (1945).

15) E. E. Snell and H. K. Mitchell, *Proc. Natl. Acd. Sci.*, **27**, 1 (1941).

16) E. E. Snell et al., *J. Bact.*, **38**, 293 (1939); *J. Biol. Chem.*, **139**, 675; **141**, 121 (1941); **143**, 519 (1942).

17) M. S. Dunn et al., *J. Biol. Chem.*, **156**, 703 (1944).

18) B. F. Steele, *ibid.*, **177**, 533 (1949).

19) K. Kitahara and A. Nakagawa, *J. Gen. Appl. Microbiol.*, **4**, 21 (1958).

20) A. L. Damain et al., *J. Bact.*, **78**, 839 (1959).

21) Kenji Sakaguchi, in preparation.

TABLE I. VITAMIN AND BASE REQUIREMENTS OF *Ped. soyae*

	d 2			d 8			dl 2		
Eliminated vitamins	O.D. 5 days	O.D. 8 days	Acid produced (N/20 NaOH ml)	O.D. 5 days	O.D. 8 days	Acid produced (N/20 NaOH ml)	O.D. 5 days	O.D. 8 days	Acid produced (N/20 NaOH ml)
none	0.40	0.37	1.60	0.33	0.44	2.14	0.41	0.37	1.75
adenine sulphate	0.40	0.39	1.79	0.35	0.43	2.18	0.39	0.43	2.15
guanine·HCl	0.42	0.43	1.60	0.33	0.43	2.21	0.43	0.45	1.95
uracil	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.73
xanthine	0.41	0.43	1.68	0.31	0.37	1.82	0.45	0.47	2.00
riboflavine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
thiamine·HCl	0.38	0.39	1.55	0.33	0.44	2.26	0.38	0.42	1.84
PABA	0.39	0.40	1.56	0.33	0.45	2.25	0.40	0.43	1.92
pyriodoxine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ca-*dl*-pantothenate	0.01	0.02	0.40	0.00	0.00	0.00	0.00	0.00	0.00
nicotinic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
biotin acid	0.14	0.12	0.84	0.07	0.04	0.23	0.13	0.13	0.78
folic	0.40	0.41	1.55	0.32	0.41	2.11	0.43	0.45	1.93
inositol	0.33	0.37	1.50	0.30	0.37	1.85	0.40	0.43	2.00
leucovorin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
choline·HCl	0.35	0.39	1.61	0.39	0.42	2.25	0.39	0.43	1.97
glycine-betaine·HCl	0.20	0.28	1.20	0.07	0.16	1.05	0.15	0.24	1.04

with biotin and glycine-betaine. However, strain d 2 required pantothenic acid and strain dl 2 required uracil only stimulatorily. In Part III of this series it was reported that *Ped. soyae* does not require leucovorin. This was proved to be errorneous in this present experiment. The error is thought to have occurred from the inevitable use of incomplete synthetic media at that time.

When the vitamin and base requirements are compared between *Ped. soyae* and salt sensitive *Ped. pentosaceus* Mees[5,10] (=*Ped. cerevisiae* Pederson[7~9]. Its nutritional study has been made by Jensen and Seeley[11].) general tendencies such as the necessity for leucovorin agreed well but stimulatory requirements for uracil, riboflavine and pyridoxine of *Ped. pentosaceus* changed more strictly to essential requirements in the case of *Ped. soyae.* This phenomenon will be infered later in a subsequent report.

As stated later, *Ped. acidilactici*, also have the same tendency in spite that they do not require leucovorin and uracil.

II Amino Acid Requirements of *Ped. soyae*

Although vitamin requirements of *Ped. soyae* had a tendency resembling other salt-sensitive pediococci, their amino acid requirements were quite different. From experiments later described and other reports[12~16] it became clear that the salt tolerant pediococci needs almost all of the amino acids for growth, but salt tolerant pediococcus *Ped. soyae* requires only glutamic acid, arginine, histidine, isoleucine, phenyl alanine, valine, tryptophane and leucine essentially, and methionine, cystine and serine stimulatorilly. The organism does not require alanine, aspartic acid, lysine, proline, threonine, tyrosine and glycine in case of the single omission experiment (Table II). The requirement for phenyl alanine is only exhibited by strain d 2.

It is noteworthy that salt tolerant pediococci were proved to more complex vitamin while more simple amino acids than salt sensitive pediococci are required.

III The Minimum Synthetic Medium for *Ped. soyae* Strain d 2

In order to exhibit the minimum synthetic medium for *Ped. soyae*, a medium composed exclusively of required vitamins, bases and amino acids manifested by the above described two experiments were prepared with the addition of glucose and salts (primary medium; the con-

TABLE II. AMINO ACID REQUIREMENTS OF *Ped. soyae*

Eliminated amino acids	d 2			d 8			dl 2		
	O.D.		Acid produced	O.D.		Acid produced	O.D.		Acid produced
	5 days	8 days	(N/20 NaOH ml)	5 days	8 days	(N/20 NaOH ml)	5 days	8 days	(N/20 NaOH ml)
none	0.40	0.37	1.60	0.33	0.44	2.14	0.41	0.37	1.75
DL alanine	0.37	0.36	1.64	0.27	0.34	2.10	0.42	0.43	2.00
DL aspartic acid	0.37	0.38	1.50	0.26	0.40	2.13	0.34	0.42	1.87
L glutamic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L arginine·HCl	0.01	0.03	0.46	0.01	0.01	0.28	0.01	0.01	0.40
L lysine·HCl	0.34	0.31	1.41	0.25	0.32	1.75	0.28	0.34	1.70
L histidine·HCl	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.19
DL isoleucine	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
DL methionine	0.18	0.20	1.14	0.19	0.24	1.49	0.19	0.20	1.16
DL phenylalanine	0.00	0.00	0.00	0.31	0.41	2.00	0.29	0.35	1.70
L proline	0.36	0.35	1.55	0.32	0.37	1.95	0.30	0.38	1.94
DL threonine	0.30	0.30	1.40	0.20	0.30	1.80	0.28	0.32	1.70
L tyrosine	0.37	0.35	1.63	0.25	0.34	1.75	0.30	0.35	1.79
DL valine	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.11
DL tryptophane	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L cystine	0.19	0.21	1.25	0.00	0.22	1.45	0.11	0.16	1.25
DL serine	0.22	0.23	1.10	0.00	0.20	1.40	0.16	0.20	1.20
glycine	0.36	0.34	1.51	0.16	0.38	1.82	0.29	0.33	1.75
L leucine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

stituents were, uracil, riboflavine, pantothenic acid, nicotinic acid, leucovorin, pyridoxine, biotin, glycine-betaine, glutamic acid, arginine, histidine, isoleucine, phenylalanine, valine, tryptophane, leucine, methionine, cystine, serine, glucose and salts.). However, on this medium, no grown of *Ped. soyae* strain d 2 was observed. After repeated experiments of supplementing other nutrients, normal growth of the strain d 2 was exhibited on the medium supplemented with xanthine, folic acid, thiamine, aspartic acid and threonine on the primary medium (Table III). It is thus considered that these supplemented vitamins and amino acids can be synthesized in the cell of *Ped. soyae* when one of each nutrient is singly eliminated from the basal complete medium though they can not be synthesized from nutrients constructing the primary medium. Therefore, it is presumed that biosynthesis of the five supplemented nutrients is accomplished by the metabolic action of one or more of these nutrients.

TABLE III. GROWTH OF *Ped. soyae* d 2 ON THE MINIMUM SYNTHETIC MEDIUM

	O.D.	Acid produced (N/20 NaOH, ml)
Basal medium	0.299	3.40
Medium composed of essential and stimulatory nutrients on Table I and II	0.00	0.00
+ xanthine, folic acid, thiamine, aspartic acid and threonine	0.267	2.84

Assays were made after five days' culture.

IV Vitamin and Amino Acid Requirements of *Ped. acidilactici* Kitahara's strain and *Ped. pentosaceus* Kitahara's strain

Ped. acidilactici Kitahara's strain and *Ped. pentosaceus* Kitahara's strain were orignally isolated from corn mashes incubated at 40°C by Kitahara[5,6] in 1940, and then identified as *Ped. lindneri* Henneberg and *Ped. hennebergi* Sollied, respectively. Taxonomical studies were recently resumed and the new species names were bestowed.

On examining the nutritional requirements of the two strains, it was observed that the requirements of *Ped. pentosaceus* Kitahara's strain had identical vitamin, base and amino acid requirements with *Ped. cerevisiae* Pederson (=Mees[5,10]) which was determined by Jensen

TABLE IV. VITAMIN AND BASE REQUIREMENTS OF *Ped. pentosaceus* KITAHARA'S STRAIN AND *Ped. acidilactici* KITAHARA'S STRAIN

	Ped. pentosaceus		*Ped. acidilactici*	
Eliminated vitamins	O.D.	Acid produced (N/20 NaOH ml)	O.D.	Acid produced (N/20 NaOH ml)
none	1.22	6.47	0.28	1.55
adenine sulphate	1.24	6.73	0.27	1.15
guanine·HCl	1.20	6.65	0.29	1.35
uracil	1.26	6.78	0.27	1.40
xanthine	1.24	6.40	0.31	1.65
riboflavine	1.26	6.72	0.00	0.20
thiamine·HCl	1.24	6.43	0.24	1.26
PABA	1.12	6.00	0.31	1.66
pyridoxine	1.18	6.49	0.00	0.00
Ca-*dl*-pantothenate	0.05	0.68	0.05	0.40
nicotinic acid	0.05	0.00	0.00	0.00
biotin	0.07	0.50	0.04	0.30
folic acid	1.20	6.40	0.29	1.48
inositol	1.24	6.61	0.29	1.55
leucovorin	0.05	0.45	0.34	1.30
choline·HCl	1.24	6.60	0.37	1.94
betaine·HCl	1.20	6.40	0.36	2.05

Assays were made after three days' culture.

TABLE V. AMINO ACID REQUIREMENTS OF *Ped. pentosaceus* KITAHARA'S STRAIN AND *Ped. acidilactici* KITAHARA'S STRAIN

	Ped. pentosaceus		*Ped. acidilatici*	
Eliminated amino acids	O.D.	Acid produced (N/20 NaOH ml)	O.D.	Acid produced (N/20 NaOH ml)
none	1.22	6.47	0.28	1.55
DL alanine	0.00	0.00	0.05	0.26
DL aspartic acid	0.01	0.00	0.00	0.00
L glutamic acid	0.00	0.00	0.00	0.00
L arginine·HCl	0.02	0.21	0.00	0.30
L lysine·HCl	0.67	4.23	0.00	0.00
L histidine·HCl	0.00	0.00	0.00	0.00
DL isoleucine	0.00	0.00	0.00	0.00
DL methionine	0.45	2.35	0.31	1.80
DL phenylalanine	0.12	0.90	0.09	0.45
L proline	0.00	0.00	0.00	0.00
DL threonine	0.00	0.00	0.00	0.00
L tyrosine	0.02	0.00	0.00	0.70
DL valine	0.00	0.00	0.00	0.00
DL tryptophane	0.00	0.00	0.00	0.00
L cystine	0.12	1.02	0.19	0.25
DL serine	0.45	2.04	0.00	0.00
glycine	0.00	0.00	0.00	0.00
L leucine	0.00	0.00	0.00	0.00

Assays were made after three days' culture.

and Seeley in 1954[11] (Tables IV and V). It is a marvelous fact that only morphologically resembling strains isolated from such apart places and in different ages have such uniform nutritional requirements. The requirements were as follows; purines and pyrimidines not required, leucovorin, pantothenic acid, nicotinic acid, biotin and all amino acids required. Of these requirements, biotin, lysine, methionine and serine were stimulatory. The identification of this strain *Ped. pentosaceus* was thus also confirmed from the view point of nutritional studies.

Vitamins necessary for *Ped. acidilactici* Kitahara's strain were, riboflavine, pyridoxine, pantothenic acid, nicotinic acid and biotin, while purines, pyrimidines or especially, leucovorin was not needed. This independency on leucovorin is a distinct character of *Ped. acidilactici* differentiating the species from other pediococci.

These nutritional specificities of this strain closely resemble the results obtained from other strains of the same species like so-called *Leuconostoc mesenteroides* P-60[14~16]. Nakagawa and Kitahara's strain reported that *Ped. acidilactici* required riboflavine, nicotinic acid, a pantothenic acid, biotin but does not require pyridoxine. These results almost agree with these of the author's, with the exception of the unnecessity of pyridoxine. This discrepancy might be due to their long culture period or to the basal medium containing casamino acids as the amino acid source.

Ped. acidilactici Kitahara's strain requires all the amino acids except methionine. For strain P-60, the requirements for all the amino acids except alanine were reported by Dunn[17] and Steele[18], and this character made this strain useful for bioassay of amino acids among other many species of lactic acid bacteria.

This character requiring almost all amino acids for growth is the specificity of salt-sensitive pediococci, both *Ped. acidilactici* and *Ped. pentosaceus*, which differentiate them from other lactic acid bacteria and even salt-tolerant pediococci. This phenomenon suggests that these two species do not have transaminase, or are not activated even in keeping the enzyme in the cell. The existence of activated transaminase in cells of salt-tolerant pediococci is an interesting problem deserving future research.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 644~646, 1960]

Studies on the Sunlight Flavor of Beer

Part VII. Correlation of the Occurrence of the Sunlight Flavor of Beer to Humulone, Lupulone and Related Compounds (2)

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The sunlight flavor occurred when humulone or lupulone was added to beer after main fermentation was completed, and among the other phenols used, only acetolupuphenone [1-acetyl-3,3,5-(γ,γ-dimethylallyl)-cyclohexadiene-(1,5)-diol-(2,6)-on(4)] was found to cause the sunlight flavor.

In part V[1] of this series, the conditions for the occurrence of the sunlight flavor of beer were established. There were four essential factors related to the occurrence of the sunlight flavor:

1. Wort or sucrose solution, the former being able to be substituted by the latter and other fermentable carbohydrates
2. Addition of humulone or lupulone
3. Main fermentation attributed to yeast
4. Visible light at the wave length during 400~450 mμ.

It was obscure, however, how these four factors are connected with the sunlight flavor of beer. In this report, attempt was made to investigate the action of these factors.

Since the addition of humulone or lupulone was characteristic to beer brewing compared with other alcoholic beverages, these bitter substances of hop might be the most important factor among the above four conditions. For instance, the sunlight flavor has not occurred from sake which was brewed by similar way but only different from beer in lack of addition of hop.

Hitherto, humulone or lupulone was added to wort or sucrose solution before main fermentation attributed to beer yeast. If degradation of humulone or lupulone by enzyme of yeast during main fermentation is not related to the occurrence of the sunlight flavor, in the case of beer which humulone or lupulone was added after main fermentation the sunlight flavor will occur. From this standpoint, the brewing method of beer to be tested was slightly modified in this experiment. Ten percent solution of sucrose was boiled for one hour, after cooling, beer yeast was added to this solution and main fermentation was completed. To this fermented solution, humulone, lupulone or other materials to be tested were added respectively and each mixture was exposed to sunlight for about six hours, and then an organoleptic test was carried out. Similar experiment was carried out for commercial sake. These results are shown in Table I.

It is observed that the sunlight flavor occurs in case of that humulone or lupulone which was added to beer after main fermentation was completed. Since the typical sunlight flavor of beer also occurs from sake by the addition of humulone of lupulone, it is cleared that humulone and lupulone are the main factors for the occurrence of the sunlight flavor of beer. Moreover, from the fact that the sunlight flavor occurs when humulone or lupulone was added to the fermented

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[1] Y. Obata and H. Horitsu, *Bull. Agr. Chem. Soc. Japan*, **22**, 153 (1958).

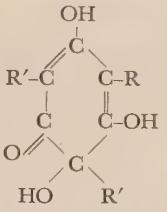
TABLE I. OCCURRENCE OF THE SUNLIGHT FLAVOR OF BEER IN THE CASE OF ADDITION OF HUMULONE OR LUPULONE AFTER MAIN FERMENTATION

Samples	Exposure[a]	Sunlight Flavor[b]
Fermented Solution	+	×
〃 + Humulone	+	0
〃 + 〃	−	×
〃 + Lupulone	+	0
〃 + 〃	−	×
〃 + Humulone[c]	+	0
〃 + Lupulone[c]	+	0
Sake	+	×
〃 + Humulone	+	0
〃 + Lupulone	+	0

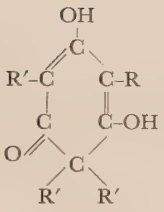
a). + ; Sunlight exposure, − ; no exposure
b). × ; Non-occurrence of sunlight flavor, 0 ; Occurrence of sunlight flavor
c). Fermented solution was heated at 70°C for one hour. Twenty mg of humulone or lupulone was added to 180 ml of fermented solution and sake.

solution which was previously heated, enzymic action to these bitter substances may be not related to the occurrence of the sunlight flavor. From these results, it is suggested that the sunlight flavor of beer occurs by a photochemical reaction between humulone or lupulone and one or more than two components present in the fermented solution, though there was no evidence what the latter components were. Further investigation on this substances is now under achievement.

Next, in connection with the structures of humulone (Ia) and lupulone (IIa), some simple phenols were examined for the occurrence of the sunlight flavor. In this experiment, catechol, resorcinol, hydroquinone, phloroglucinol and phloroacetophenone were used. These phenols were added to the fermented solution (20 mg/180 ml) and the mixture was exposed to sunlight, but in all cases the sunlight flavor of beer is not detected. This fact shows that some specific structural components other than phenolic group seem required for the occurrence of the sunlight flavor.

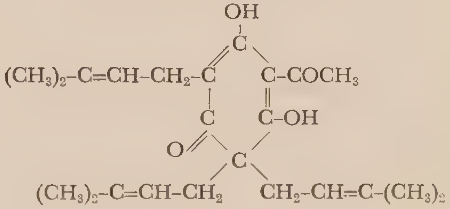


I

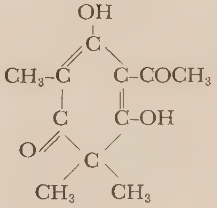


II

R′= $(CH_3)_2 \cdot C{=}CH \cdot CH_2$-
Ia, IIb, R= $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CO$-
Ib, IIb, R= $(CH_3)_2 \cdot CH \cdot CO$-
Ic, IIc, R= $CH_3 \cdot CH_2 \cdot (CH_3) \cdot CH \cdot CO$-



III



IV

In the previous report[2], both cohumulone(Ib) and adhumulone(Ic) were found to be the cause of the sunlight flavor as well in the case of humulone. The chemical structures of cohumulone and adhumulone only vary at the acyl rest of humulone. It seems likely, therefore, that the acyl rest of humulone or lupulone is not related to the occurrence of the sunlight flavor, though we have not examined on lupulone analogues, i.e., colupulone(IIb) and adlupulone(IIc). For the purpose of this, lupulone analogue which has a simpler acyl rest, acetolupuphenone(III) 〔1-acetyl-3,3,5-(γ,γ-dimethylallyl)-cyclohexadiene-(1,5)-diol-(2,6)-on-(4)〕 was synthesized. Acetolupuphenone has not yet been found in natural sources, it is a synthetic lupulone analogue. This was synthesized according to Riedl's method[3] using phloroglucinol as the starting material. Thirty mg of acetolupuphenone were added to the above fermented solution (180 ml) and the mixture was exposed to sunlight for six hours. The typical sunlight flavor of beer was detected as was expected. As mentioned above, it is cleared that the acyl rests of humulone and lupulone are not related to the sunlight flavor.

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[2] Y. Obata and H. Horitsu, *Bull. Agr. Chem. Soc. Japan*, **23**, 186 (1959).

[3] W. Riedl, *Chem. Ber.*, **85**, 692 (1952).

In the previous report, it was found that 3-methyl-5-acetyl-filicinic acid(IV) did not cause the sunlight flavor. This compound was reexamined under this modified experimental condition. But, the sunlight flavor is not detected in this case. 3-Methyl-5-acetyl-filicinic acid resembles to the structures of lupulones, especially acetolupuphenone, but has a simple side chain, i. e., the methyl group instead of prenyl(γ,γ-dimethylallyl) group of lupulones. From the facts that acetolupuphenone causes the sunlight flavor but 3-methyl-5-acetyl-filicinic acid does not, it is supposed that the prenyl group in the molecules of humulone and lupulone is closely correlated to the occurrence of the sunlight flavor of beer. Further investigation is proceeding in this laboratory.

EXPERIMENTAL

Isolation of Humulone and Lupulone

Hops were extracted with petroleum ether according to the method of Lewis et al.[4], and humulone and lupulone were isolated from the petroleum extract as usual way.

Phenols

Catechol, resorcinol, hydroquinone and phloroglucinol were purchased. Phloroacetophenone was synthesized from phloroglucinol according to the method of Hoesch[5]. 3-Methyl-5-acetyl-filicinic acid was formerly synthesized in this laboratory by the method of Riedl[6].

Synthesis of Acetolupuphenone (III) [1-Acetyl-3,3,5-(γ,γ-dimethylallyl)-hexadiene-(1,5)-diol-(2,6)-on-(4)]

This was synthesized from phloroglucinol according to the method of Riedl. It was recrystallized from methanol and the crystalline gave m.p. 119~120°C. Found: C, 74.26; H. 8.25: Calcd for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66%. The UV-absorption spectrum of III are Fig. 1 and Fig. 2.

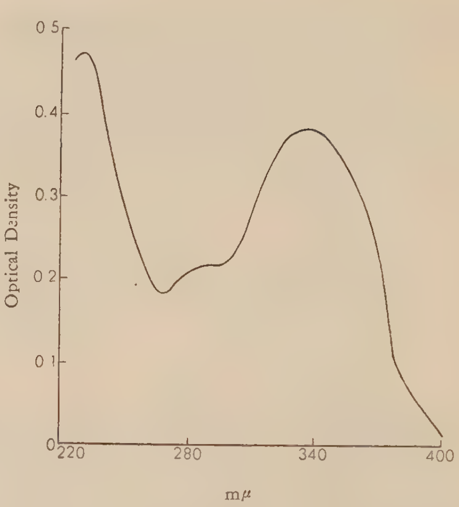

FIG. 1. Absorption Spectrum of Acetolupuphenone (in Ethanol).

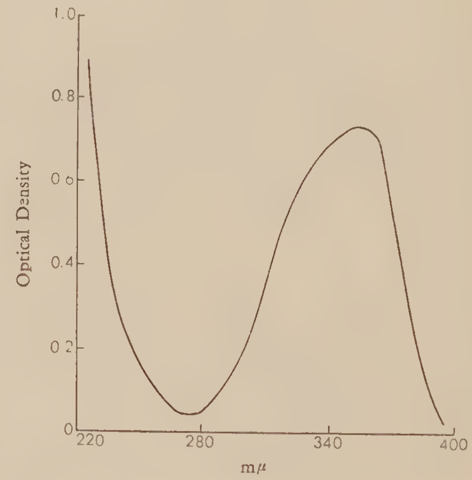

FIG. 2. Absorption Spectrum of Acetolupuphenone (in Alkaline Methanol Solution).

Examination of Sunlight Flavor

The percent solution of sucrose was boiled for one hour. After cooling, well washed beer yeast was added to this and main fermentation was carried out at 0~5°C for ten days. This fermented solution was removed into the sterilized brown bottles and stored in the refrigator. In case of examination of sunlight flavor, aliquots of this stored solution were put into the sterilized colorless bottles. To these each test substance was added, respectively. The mixture was exposed to sunlight for about six hours and organoleptic test was made.

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[4] J. C. Lewis, G. Alderton, J. F. Carson, D. M. Reynolds and W. D. MacLay, *J. Clin. Invest.*, **28**, 916 (1949).

[5] K. Hoesch, *Ber.*, **48**, 1122 (1915).

[6] W. Riedl and K. H. Risse, *Liebigs Ann. Chem.*, **585**, 209 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 647~651, 1960]

A New Toxic Substance, Teleocidin, Produced by *Streptomyces*.

Part I. Production, Isolation and Chemical Studies

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Received May 11, 1960

In the course of studies on the screening of specific toxic substances produced by *Streptomyces* against aquatic organisms, it was recognised that a newly isolated *Streptmyces*, 2A 1563, which was considered to be a variant species of *Streptomyces mediocidicus* produces a new toxic substance, Teleocidin. Teleocidin was isolated as a white powder resembling crystals with a crystal appearance from the methanol extracts of the cultured mycellium, and showed a specific toxic action toward Japanese killifish and mice, but did not exhibit any inhibition against microorganisms.

From our experience it was found that the mycellial component produced by a strain belonging to *Streptomyces* assumes a specific toxicity to fish, and we are now setting ourselves about to investigate the utilization of such toxic action.

Primary screening methods for measurement of the toxicity of antibiotics or biologically active substances have been reported by many workers. Seneca et al.[1] have suggested the use of snails for initial screening of the toxicity of antibiotics, Mengle et al.[2] have proposed the use of cockroaches as test animals for antibiotic screening, Ballard et al.[3] the use of goldfish in screening of water-soluble drugs for neurotoxicity, Turner et al.[4] the use of daphnia for screening of toxicity of antibiotics, Umeda et al.[5] the use of Japanese killifish in the screening of cardiotonics and Hoshi[6] the use of the ova of pig ascaris for screening of anthelmintics. From such instances it was considered that the use of fishes as test animals has merit in the screening of such toxic substances to aquatic organisms, e.g., barnacles and bryozoa.

From the results of screening of such toxic substances produced by *Streptomyces* against Japanese killifish (*Oryzias latipes*), using daphnia, nematoda and insects as test animals it was found that several strains produce highly toxic substances in *Oryzias latipes* and daphnia. One of these substances, substance[7] 2A 720, was revealed to be indentical with Antimycin A originally reported by C. Leben et al.[8]. Thereafter, a new toxic substance having high toxicity toward fishes has been isolated from the cultured mycellium of a newly isolated *Streptomyces* 2A 1563. This toxic substance which we named Teleocidin did not exhibit any inhibition against microorganisms, whereas it exhibited strong toxicity to aquatic organisms similarly to mammalians and their skin.

This paper deals with taxonomic studies of this strain, fermentation, isolation and certain chemical properties of this toxic substance. The biological activities of Teleocidin will be the subject of a subsequent report.

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1) H. Seneca and E. Bergendahl, *Antib. and Chemoth.*, **5**, 737 (1955).

2) D. C. Mengle and F. W. Fisk, *ibid.*, **6**, 607 (1956).

3) B. E. Ballard, J. Dufrenoy and R. Pratt, *J. Am. Pharm. Assos.*, **45**, 181 (1956).

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EXPERIMENTAL AND RESULTS

Teleocidin producing strain

Morphological and physiological characters were studied on the media described in this section. The most characteristic morphological form of aerial mycellium on various medium is whirl formation. Primary or secondary whirls were observed respectively in accordance with conditions of cultivation and the medium used. Observations of this strain on various media are presented in Table I. Utilization of the carbon sources was tested using Pridham's solution as the basal medium at 28°C. Glucose, trehalose and inositol were easily utilized, and arabinose was slightly utilized. Xylose, rhamnose, lactose, sucrose, raffinose, mannitol and salicin were not utilized.

TABLE I. CULTURAL PROPERTIES OF *Storeptomyces* 2A 1563 (at 28°C).

medium	growth	aerial mycellium	soluble pigment
bouillon agar	dark brown, slightly dried surface	scant, white, short hyphae	none
glucose asparagine agar	pale brown slightly spread	white, thin, short hyphae	none
Czapek's agar	pale yellowish brown, colonial	white, short hyphae whirl formation	none
starch ammonium agar	yellow, fair growth*	yellowish white, long hyphae whirl formation	none
glucose Czapek's sol.	white, mass at the bottom**	no developed	none
glucose bouillon sol.	white mass at the bottom***	no developed	slightly brown
potato plug	cream colored yeast like colony, fair growth	no developed	none
gelatin	fair growth at the bottom of liquified portion, with quick liquifaction****	no developed	none

* : hydrolysis moderate
** : marked nitrite formation
*** : pH, almost no change
**** : at 20°C, for 15 days

The antimicrobial spectrum of *Streptomyces* 2A 1563 was tested by the agar disk method. Fungi and yeasts, for instance, *Aspergillus niger*, *Penicillium chrysogenum*, *Rhizopus nigricans*, *Saccharomyces cerevisiae*, *Tolura utilis* and *Candida albicans* were strogly inhibited and *Mycobacterium* and Gram positive bacteria were also inhibited, while Gram negative bacteria were not inhibited or only slightly inhibited.

The acetone extracts of the shaking cultured mycellium showed an antifungal activity, and from its charcteristic UV absorption spectrum it was suggested that this antifungal agent is a hexaene antifungal antibiotic.

Among numerous species of *Streptomyces* described in Bergey's Determinative Bacteriology (1957) and appearing in many other literature, *Streptomyces mediocidicus*[9] appears to be most closely resembled to the *Streptomyces* 2A 1563 in respect of whirl formation on aerial mycelia, production of the hexaene antifungal antibiotic and other characteristics. However, this strain differs from *S. mediocidicus* in the following respect; the production of brown colored soluble pigment on protein containing medium is either very weak or no pigment is produced at all.

This strain is therefore considered to be a variant species of *Streptomyces mediocidicus*.

Production of Teleocidin

Cultural conditions were studied using a reciprocating shaking machine. One hundred ml of a medium was poured onto a 500 ml flask (volume) and 2 drops of soy bean oil were added as an antifoam agent. After sterilization, 3 to 5 loopfuls of amounts grown on Bennet's agar slant were inoculated to the test medium and shake cultured at 28°C. The following medium was established to be suitable for production; 2% glucose, 1% soy bean meal, 0.1% meat extract, 0.5% ammonium sulfate, 0.25% Ebios (dried brewer's yeast), 0.02% potassium monohydrogen phosphate, 0.4% potassium chloride, 0.4% calcium carbonate and pH adjusted to 8.0 prior to sterilization.

Deep aerated fermentation was made, using jar fermentors of 10 l capacity. Eight liters of the medium was placed in a fermentor and sterilized for twenty minutes at 120°C. Five hundred ml of the forty-eight hours shake-cultured broth described above was inoculated. Cultural conditions were as follows; temperature 28°C, pH 6.8 to 5.6, stirring 250 r.p.m., and air 8 l per minute.

From seventy-two to ninety-six hours after cultivation, maximum toxic activity was observed and the toxic substance was produced in almost all of the mycellium.

For testing toxicity of the cultured broth or methanol extracts of mycellium, adult *Oryzias latipes* (Japanese

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9) Y. Okami, R. Utahara, S. Nakamura and H. Umezawa, *J. Antibiotics (Japan)*, Ser. A 7, 98 (1954).

killifish, Himedaka) was used as the test organism and Antimycin A as a standard sample. The same technique as described by Umeda et al.[5] was adopted because some parallelism between the toxicity to mice and fishes was found in the preliminary test, and also because this method was considered to be much easier and less expensive. In purification studies, the irritative test for rabbit skin and colorimetric determination involving the diazo reaction were used subsidiary.

At the end of fermentation the toxicities of methanol extracts of mycellium and the filtered broth to fishes gave the following results: Solution of methanol extracts of mycellium diluted one-thousand times caused complete death within one hour and solutions diluted ten thousand times caused complete death within twenty-four hours, but the filtered broth solutions diluted one hundred times did not show any influence to fishes within twenty-four hours.

Isolation and purification of Teleocidin

Sixteen liters of cultured broth was filtered with 400 g of hyflosupercel. The filtered cake was eluted with two 5-l portions of methanol for two hours from 45 to 50°C. The combined brown colored eluates were concentrated to 200 ml in vacuo and extracted with four 500-ml portions of ethyl ether. These ether extracts were then combined and dried on sodium sulfate, and thereafter evaporated. The blackish brown oily syrup thus obtained was purified chromatographically on active alumina column repeatedly. At first, the toxic fraction was absorbed on alumina column from ether solution and eluted with ether and acetone. Finally, the toxic substance was absorbed on alumina from *n*-hexane-benzene (4 : 1) solution and eluted with ethyl-acetate and acetone, and, after evaporation of the solvents under vacuum and drying, 2.5 g of brown colored crude powder was obtained.

Further purification was achieved by repeated application of the counter current distribution method and absorption chromatography on active alumina and anhydrous silicic acid. After using the Craig's apparatus several times with the following solvent system: benzene-*n*-hexane-methanol-water (1.5 : 9 : 10 : 2.5) or (2 : 9 : 10 : 2.5) and chloroform-methanol-water (10 : 7 : 1), a distribution curve was obtained by 240 transfers using 80 tubes of Craig's apparatus (solvent system: benzene-*n*-hexane-methanol-water, 1.5 : 9 : 10 : 2.5) at room temperature as shown in Fig. 1. The result of this experiment showed that a peak appeared at tube No. 61 and the distribution coefficient of this toxic substance was 0.341. The theoretical curve calculated from the distribution coefficient is also shown in Fig. 1. It coincided with the experimental curve elucidating homogeneity of the toxic powder obtained here. The purified powder collected from the distribution tubes was absorbed on an anhydrous silicic acid column from *n* hexane-benzene-ethylacetate (4 : 1 : 1) solution and eluted as a single band under UV light with benzene-ethylacetate mixture from the column, and thereafter evaporated and dried. The yield of the powder thus obtained was ca 0.5 g from 2 g of crude powder. The colorless powder thus obtained by paperchromatography on Toyo filter paper No. 50 using ethanol-glacial acetic acid-water (12 : 1 : 11) and benzene-*n* hexane-methanol-water (2 : 8 : 10 : 2.5, lower layer) afforded only a brown coloured single spot by ferric chloride solution or Dragendorf's reagent at R_F 0.664 and 0.873 at room temperature, respectively.

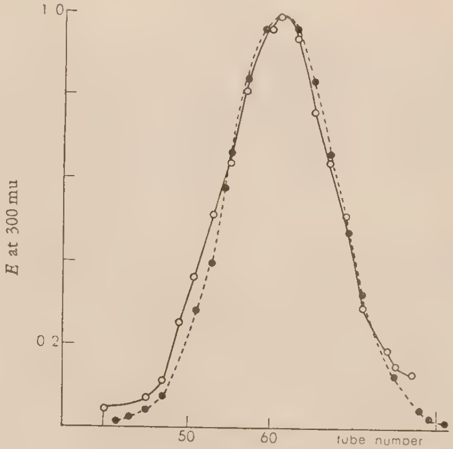

FIG. 1. Counter Current Distribution of Teleocidin.

Solvent system: benzene-hexane-methanol-water (1.5 : 9 : 10 : 2.5). 80 tubes 240 transfers.
○——○ experimental, ●----● theoretical,
max tube, No, 61, K=0.341.

Physical and chemical properties of Teleocidin

Teleocidin is a white crystal like powder and does not show a definite melting point but gradually melts with decomposition above 61°C. It does not contain sulfur, phosphorus, halogens, ashes and the methoxyl group. Results of elementary analysis which as yet, do not give a constituent value were as follows: C, 73.31; H, 9.17; N, 7.70% and molecular weight measurements by Rast's and Barger-Akiya's methods gave 279.318 and 282.333 respectively. The ultraviolet absorption spectrum in methanol solution is indicated in Fig. 2. The absorption maxima were 229∼231, 286∼294 and 298∼302 mμ. The infra-red absorption spectrum in nujol is also given in Fig. 3.

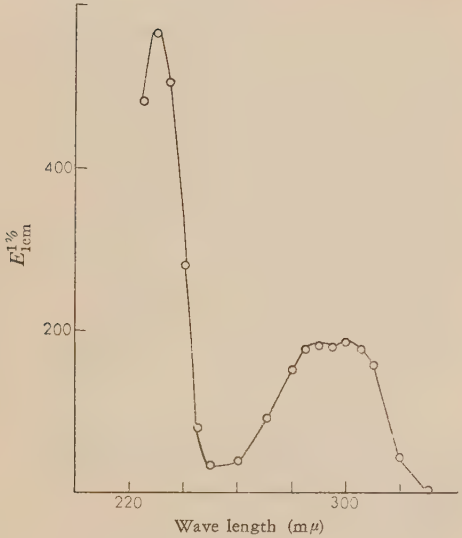

FIG. 2. Ultraviolet Absorption Spectrum of Teleocidin (in methanol).

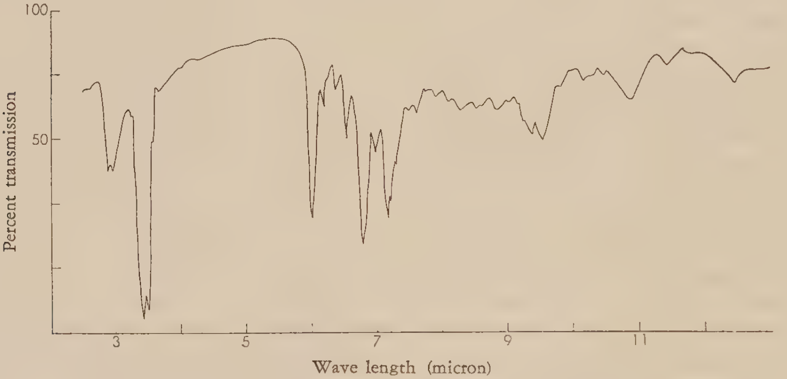

FIG. 3. Infra Red Absorption Spectrum of Teleocidin (in nujol).

Teleocidin is easily soluble in methanol, ethanol, ether, acetone, ethyl-acetate, benzene, chloroform, carbon tetrachloride and only slightly soluble in petroleum-ether, petroleum-benzine and ligroin. It is almost insoluble in water, 5% hydrochloric acid and 5% sodium hydroxide solution. It gives a green color in methanol solution and a brown color on the paper spot with ferric chloride solution. It also gives positive diazo, phosphomolibdate, Tollen's, Dragendorf's, pyrrol, fluorescein chloride, brom, Baeyer's and concentrated sulfric acid reactions, but negative ninhydrin, biuret, Fehling's, Ehrlich's and 2,4-dinitrophenylhydrazine reactions. Teleocidin is very stable in organic solvents and relatively stable in alkaline solution, showing almost no loss in toxicity in the pH range of 7 to 9 by heating for ten minutes at 100°C; however after standing for one month at 37°C, it becomes rather unstable in acidic solution.

Biological properties of Teleocidin

Teleocidin showed a specific toxic action toward fishes and other aquatic organisms equally to mammalians, while it did not exhibit any inhibition against bacteria, molds, yeasts, diatoms, protozoa, Ehrlich's carcinoma and plant seeds up to the concentration of 100 μg per ml. The toxicity of Teleocidin to aquatic organisms will be reported in a subsequent paper. Toxicity of Teleocidin was tested in mice weighing from 15 to 20 g. The LD 50 by intravenous injection was 0.28 for 0.40 mg per kg and LD 50 by oral administration 2 mg per kg.

DISCUSSION

Among some known antibiotics and toxic substances produced by *Streptomyces* species, Actinomycins[10], Actinoleukin[11], Antimycin A[8], Carzinophillin[12], Xanthomycin[13] etc. has been reported as highly toxic antibiotics to mammalian, and Azomycin[14], Actidion[15] and Cytovirin[16)] as skin irritative substances, and Oligomycin[17)] as a soil nematoda cidal substance, and Antimycin A and Netropsin[18)] as toxic substances toward fishes and insects, and Ascaricidin[6)] as a toxic substance toward the ova of pig ascaris.

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[10] S. A. Waksman and H. B. Woodruff, *J. Bact.*, **42**, 23 (1941).

[11] M. Ueda, Y. Tanigawa, Y. Okami and H. Umezawa, *J. Antibiotics* (*Japan*), Ser. A **7**, 125 (1954).

[12] T. Hata, F. Koga, K. Kanamori, A. Matsumae, R. Sugawara, T. Hoshi, T. Shima, S. Ito and S. Tomizawa, *ibid.*, Ser. A **7**, 107 (1954).

[13] C. B. Thorne and W. H. Peterson, *J. Biol. Chem.*, **176**, 413 (1948).

[14] K. Maeda, T. Osato and H. Umezawa, *J. Antibiotics* (*Japan*), Ser. A **6**, 182 (1953).

[15] A. J. Whiffen, N. Bohonos and R. L. Emerson, *J. Bact.*, **52**, 610 (1946).

Our new toxic substance, Teleocidin, is clearly differentiated from these antibiotics by its non-inhibitory action against bacteria, molds and yeasts.

Aureothin[19)] is reported to have toxicity to mice and non toxicity to microorganisms, but both chemical and physical characters differ much from each other.

Although a pure crystalline form of this toxic substance has, so far not been obtained from physical chemical and biological properties it is concluded that Teleocidin is a new toxic substance produced by *Streptomyces* species.

SUMMARY

A new toxic substance, Teleocidin, was isolated from the methanol extracts of cultured mycellium of *Streptomyces* 2A 1563 resembling *Streptomyces mediocidicus*. Teleocidin has strong toxicity to mice and its chemical and physical properties were examined.

Acknowledgements The authors wish to express their sincere gratitude to Dr. K. Sakaguchi and Prof. K. Arima, Tokyo University, for their constant guidance throughout this work and to Mr. T. Fujisawa, Dr. K. Ueda and Dr. T. Fujisawa of this company for their kind encouragement. The authors indebtness is also due to members of the Microanalytical Laboratories of the Department of Agricultural Chemistry, the Institute of Applied Microbiology of Tokyo University for the microanalysis and to Mr. K. Aizawa, the Department of Agricultural Chemistry of Tokyo University, Mr. K. Yamazaki, the Central Research Institute of Japan Monopoly Corporation, Tokyo, for their assistance in infrared absorption spectra. The authors' acknowledgement is also made to Mr. T. Matsumoto for his willing cooperation.

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A New Toxic Substance, Teleocidin, Produced by *Streptomyces*.

Part II. Biological Studies of Teleocidin

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Received May 11, 1960

The isolation of a new toxic substance, Teleocidin, has been reported in a previous paper. The biological activities of Teleocidin were examined and it did not exhibit any inhibition against microorganisms, but has shown strong toxic actions toward aquatic organisms, i.e. *Oryzias latipes*, *Daphnia magna* and *Bugula neritina* larvae, at a concentration range between 1 μg to 0.05 μg/ml. Teleocidin has also shown a toxic effect toward nematoda, i.e. *Ascaris suilla* and *Turbatrix aceti*, at a concentration range between 25 μg to 10 μg/ml.

The toxicity toward mammalians and skin irritative effects of Teleocidin were also observed in the rabbit.

A new toxic substance, Teleocidin, has been isolated from the cultured mycellium of a strain of *Streptomyces mediocidicus* var. species. Studies on its production and certain physical and chemical properties of Teleocidin have been reported(1). From studies of the biological activities of Teleocidin it has been observed that Teleocidin does not exhibit any inhibition against microorganisms, but it is shown to have specific toxicity toward equatic organisms and an equal degree toward mammalians and their skin. Biological studies of this problem will be reported in this paper.

METHODS AND MATERIALS

The tested samples were prepared by dilution of a methanolic solution of Teleocidin with several volumes of water and produced fine suspensions, because Teleocidin had shown only a slight solubility of about 10 to 1 μg/ml in water. The LD 50 to mice after intravenous injections of partly purified Teleocidin which was used in all experiments ranged 0.13 to 0.59 mg/kg.

EXPERIMENTS AND RESULTS

Toxicity to *Oryzias latipes* (Japanese killi-fish, Himedaka)

Methanolic solutions were serially diluted in 50 ml of dechlorinated tap water and 5 fishes were exposed for twenty-four hours to these testing solutions in 3-inch petri dishes. In 10 to 1 μg/ml solutions all fishes completely died within two hours and in a 0.05 μg/ml solution, death occurred within twenty-four hours at room temperature.

Among the other toxic antibiotics(2) produced by *Streptomyces*, Trichomycin, Thiolutin, Rimocidin, Eurocidin, Netropsin, Actidion, Carzinophillin, Antimycin A and Actinomycin J were also tested.

The results are given in Table I. Only Antimycin A showed such severe toxicity as Teleocidin toward fishes. Potter et al.(3) and Lardy et al.(4,5) have reported that Antimycin A has great affinity for one component of the electron transport system, so we have compared their effect on oxidations by use of liver homogenates between Teleocidin and Antimycin A. The results are shown in Table II. Blastmycin, one of the components of Antimycin A, affected this system, while Teleocidin and SK-toxic substance, one of the analogous substances of Teleocidin produced by an another *Streptomyces*, did not prove to be active in this test. It thus seems likely that Teleocidin and Antimycin A would act as inhibitors of a different type.

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1) M. Takashima and H. Sakai, This Bulletin, **24**, 647 (1960).

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3) V. R. Potter and A. E. Reif, *J.B.C.*, **194**, 287 (1952).

4) H. A. Lardy, D, Johnson and W. C. Murray, *Arch. Biochem. and Biophy.*, **78**, 587 (1958).

5) H. A. Lardy and H. Wellman, *J.B.C.*, **195**, 215 (1952).

TABLE I. TOXICITY TO *Oryzias latipes* AND *Daphnia magna* OF TELEOCIDIN AND TOXIC ANTIBIOTICS PRODUCED BY *Streptomyces*. (at 28°C)

substances	toxicity to *Oryzias latipes* in μg/ml* 1 hr	24 hrs	toxicity to *Daphnia magna* in μg/ml* 72 hrs
Trichomycin	100	10	1
Thiolutin	100	100	10
Rimocidin	100	10	100
Eurocidin	100	10	10
Netropsin	100	100	10
Actidion	100	100	0.1
Carzinophillin	100	10	1
Actinomycin J	10	1	0.1
Antimycin A	1	0.1	0.1
Teleocidin	1	0.1	0.1
PCP**	1	1	—
BHC**	20	20	—
DDT**	200	2	—
Parathion**	50	5	—

* the concentrations showing completely death of all test organisms.

** agricultural insecticides and disinfectant

TABLE II. EFFECT OF TELEOCIDIN ON OXIDATION BY RAT LIVER HOMOGENATES

substances	μg/2.7 ml	succinate O_2 uptake/flask/hr	glutamate O_2 uptake/flask/hr
none	—	110[1], 92[2]	58
Teleocidin	100	86	—
	10	—	56
SK-toxic subst.	25	94	—
	2.5	106	—
Blastmycin	25	0	—
	10	—	26
	2.5	0	—
	1	0	—

The reaction mixture contained 6μ moles[1] or 0.6μ moles[2] ATP, 50μ moles phosphate buffer pH 7.4, 15μ moles $MgSO_4$ and 50μ moles glucose. Hexokinase (28.000 mμ) and 0.4 ml of 10% rat liver homogenates in 0.25M sucrose were tipped from the side arm at zero time. Substrates were added in the following amounts: succinate 20μ moles, L-glutamate 30μ moles. Final volume 2.7 ml. T=30°C.

Toxicity toward *Daphnia magna*

Methanolic samples were diluted with salt solutions as reported by Turner et al.[6] and 10 to 20 daphnids were exposed to these testing solutions in 3-inch petri dishes for forty-eight hours. In a 1 μg/ml solution all daphnids were completely dead within twenty-four hours and in a 0.1 μg/ml solution death occurred within forty-eight hours at room temperature.

Among the other toxic antibiotics produced by *Streptomyces* Antimycin A, Actinomycin J and Actidion showed severe toxicity toward daphnids to the same extent as Teleocidin as shown in Table I.

Toxicity to *Bugula neritina* (larvae)

The larvae of bryozoa, one of the marine fouling organisms, were also tested for their tolerence to Teleocidin and other toxic antibiotics. About 30 larvae were exposed in testing solutions diluted with sea water. The results are presented in Table III. It was found that Teleocidin shows severe toxicity to larvae, namely in 1 to 0.1 μg/ml solutions all larvae were completely dead within twenty-four hours at room temperature, but Antimycin A exhibited severest toxicity in this experiment, showing toxicity about ten to one hundred times higher than Teleocidin.

TABLE III. TOXICITY TO *Bugula neritina* LARVAE OF TELEOCIDIN AND TOXIC ANTIBIOTICS PRODUCED BY *Streptomyces*

substance	toxicity in μg/ml after 24 hrs. at room temp. A*	B**
Trichomycin	100	10
Thiolutin	1	1
Rimocidin	10	1
Eurocidin	10	1
Netropsin	10	10
Actidion	10	10
Carzinophillin	10	1
Antimycin A	0.0001	0.00001
Actinomycin J	10	10
Teleocidin	1	0.1
PCP***	1	0.1

A* : the concentrations giving complete death

B**: the concentrations in which 50% or more larvae dead and alived larvae did not growth

*** disinfectant

Toxicity toward *Ascaris suilla*

Adult pig *Ascaris* was tested for its tolerence to Teleocidin. (a), Eleven petri dishes size, 3 inch were used, each dish containing one male *Ascaris* in 50 ml of the testing solution diluted with a modified Rocke-Ringer solution[7]. Five *Ascaris* suspended their locomotion in a 25 μg/ml solution within forty-eight hours at 37°C, but all *Ascaris* demonstrated reflective movements by steeping in hot water at 60°C. (b), Five male *Ascaris* were placed in a large petri dish filled with 250 ml of

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6) J. C. Turner and T. J. Lannon, *Proc. Soc. Exp. Biol. and Medic.*, **80**, 684 (1952).

7) Y. Kobayashi and T. Bando, *Tokyo Igaku-kai Zasshi*, **59**, 55 (1945).

the testing solution containing 25 μg/ml of Teleocidin. After forty-eight hours all *Ascaris* suspended their locomotion and did not react with the steeping in hot water. The toxicity toward pig *Ascaris* of Teleocidin and some other anthelmintics is shown in Table IV.

TABLE IV. TOXICITY TO *Ascaris Suilla* OF TELEOCIDIN AND SOME ANTHELMINTICS AFTER 48 HRS. AT 37°C**.

substance	conc. in μg/ml	Nos. of test *Ascaris*	Nos. of *Ascaris* which suspended locomotion	Nos. of dead *Ascaris*
Teleocidin	100*	—	—	—
	10	3	2	2
Kainic acid	100	3	0	0
	10	3	0	0
Hexylresorcinol	100	3	3	3
	10	3	1	0
Santonin	100	3	1	1
	10	3	0	0

* Because Teleocidin showed only slightly solubility in salts solution, the fine suspension of Teleocidin at this concentration could hardly make.

** The method (a) was used.

Toxicity to *Turbatrix aceti*

Teleocidin has been tested for its toxicity in *Turbatrix* (a vinegar nematoda). The test samples were serially diluted with a growing medium* of *Turbatrix* and numerous organisms were tested in a test tube filled with 5 ml of the testing solution. After three to four days nearly all adult organisms succumbed at the concentration of Teleocidin ranging from 0.25 to 25 μg/ml, but thereafter normal growth of *Turbatrix* was observed. A comparatively low stability of Teleocidin in acidic solution or more resistivility of the egg than adult *Turbatrix* have resulted in this experiment.

Toxicity toward mammalians and their skin

A highly toxic action of Teleocidin toward mice has previously been reported[1]. A similar high toxicity was also observed in the rabbit.

Rabbits receiving intravenous administration of 100 μg of Teleocidin per kg died within five minutes and a dose of 10 μg to 50 μg/kg of Teleocidin caused an increasing effect on blood-pressure. These toxicological and other pharmacological properties of Teleocidin will be reported in a subsequent report.

At the time of isolation and purification studies on Teleocidin, a severe skin irritative action was observed. Dry powder and its methanolic solutions at the concentration of 100 μg/ml or more caused intense hyperemia, redness of mucous membrances, formation of a puruloid exudate, necrosis and desquamation of the mouth, nose, eyes, ears and skin in the rabbit, where as when the surviving animals were sacrified no pathological changes were revealed in their organs.

Teleocidin has been tested for its toxicity of inhibitory action. Twenty different species of microorganisms, diatoms, 3 species of protozoa, Ehrlich's carcinoma, plant pathogenic virus and plant seeds were tested.

The results of these tests indicated that Teleocidin does not exhibit any inhibition up to the concentration 100 μg/ml.

DISCUSSION

The data presented in this paper indicate that Teleocidin has a specific toxicity of aquatic organisms and vertebrate, but does not have any inhibitory action to microorganisms.

Only Aureothin[8] has so far been reported as a toxic substance toward mice but is non-toxic to microorganisms produced by *Streptomyces*, while its toxicity to aquatic organisms and other pharmacological properties has not yet been reported.

However, in studies of the productions or purifications of new antibiotic some workers[9] have reported about or experienced such impurities having a strong toxicity or skin irritative effect, so the production of other toxic substances such as Teleocidin from different toxic antibiotics by cultivation of *Streptomyces* species may be expected.

From the results presented here Antimycin A, which is a highly selective and potent inhibitor[10] of an electron transport component in the succinoxidase system and in the DPN system, has shown equally or more severe toxicity than Teleocidin to aquatic organisms.

While mechanisms of the inhibitory actions of Teleocidin toward aquatic organisms have not yet been revealed, further pharmacological studies will serve in research of utilization of this toxic substance.

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* Equivalent volume mixture of Japanese Saké and vinegar was used as the growing medium of *Turbatrix* (pH 2.4 to 2.6).

[8] Y. Hirata, K. Okuhara and T. Naito, *Nature*, **173**, 1101 (1954); K. Maeda, *J. Antibiotics (Japan)*, Ser. A, **4**, 137 (1953).

[9] A. J. Whiffen, N, Bohonos and R. L. Emerson, *J. Bact.*, **52**, 610 (1946); K. Watanabe (Institute of Applied Microbiology, University of Tokyo), private communication.

[10] B. Chance and L. Smith, *Ann. Rev. Biochem.*, **21**, 687 (1952).

SUMMARY

Biological activities of a new toxic substance, Teleocidin, produced by a *Streptomyces* were examined. Among the toxic antibiotics produced by *Streptomyces*, 9 antibiotics were also tested for their toxicity toward *Oryzias latipes*, *Daphnia magna* and *Bugula neritina* larvae, and Antimycin A was found to show equal or more severe toxicity than Teleocidin.

Teleocidin has shown certain inhibitory actions to *Ascaris suilla* and *Turbatrix aceti* and also has severe toxicity and skin irritative effects on mammalians.

Acknowledgements. The authors wish to express their sincere gratitude to Dr. K. Sakaguchi and Prof. K. Arima of Tokyo University, and to Mr. T. Fujisawa, Dr. K. Ueda and Dr. T. Fujisawa of this company for their kind guidance throughout this work. Thanks are also due to Prof. T. Bando of Juntendo University, Assist. Prof. T. Hibiya, Assist. Prof. T. Yamazaki, Mr. R. Hirano of Tokyo University and members of this laboratory for their kind instruction and suggestions concerning the studies on toxicity.

They also wish to express their indebtness to Assist. Prof. H. Yonehara, Institute of Applied Microbiology, University of Tokyo, for kindly supplying the antibiotics used in this work.

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Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part XI. On the Reduction of Isopulegone*

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Received May 13, 1960

On the reduction of isopulegone with sodium in aqueous ammonia, it was found that the main product was isopulegol. This fact indicates that the double bond not in conjugation with carbonyl group is not attacked.

In the previous papers, it has been shown that saturated ketones ((−)-menthone[1], (−)-isomenthone[2], (±)-menthone[3], (±)-isomenthone[3], inverted menthone[4]), α,β-unsaturated ketones ((−)-carvone[5], (+)-pulegone[6], (±)-piperitone[7]) and cross conjugated ketone (piperitenone[8]) yielded saturated alcohols by the treatment with sodium in aqueous ammonia, i.e., a novel method of reduction employed in this series.

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* For Part X see This Bulletin, **24**, 601 (1960).

[1] H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).

[2] H. Ueda, *ibid.*, **24**, 595 (1960).

[3] H. Ueda, *ibid.*, **24**, 506 (1960).

[4] H. Ueda and S. Shimizu, *ibid.*, **23**, 380 (1959).

[5] H. Ueda and S. Shimizu, *ibid.*, **23**, 524 (1959).

[6] H. Ueda and S. Shimizu, *ibid.*, **24**, 92 (1960).

[7] H. Ueda, *ibid.*, **24**, 598 (1960).

[8] H. Ueda, *ibid.*, **24**, 601 (1960).

The present investigation was undertaken to determine the composition of the reduction product of isopulegone (β,γ-unsaturated ketone), on the same reduction as described above.

In the reduction of carvone, the carbonyl group and double bond conjugated with the carbonyl group in carvone were reduced, but the unconjugated double bond was not affected, dihydrocarveol being produced[5]. In view of this result, it is presumed that isopulegone would be reduced with sodium in aqueous ammonia to give isopulegol.

On the other hand, from the fact that isopulegone is easily converted to pulegone by the treatment with sodium-ethoxide or baryta[9], it is also possible to suppose that isopulegone would be converted by alkali in reaction medium to pulegone, which would be reduced to menthol, as reported previously[6].

The experiment described below was undertaken in order to make a choice between these alternative presumptions.

The experiment showed that the reduction product of isopulegone consisted of 90% of unsaturated alcohol and 10% of menthol. The unsaturated alcohol was characterised mainly as (−)-isopulegol by the bromide of its 3,5-dinitrobenzoate. Catalytic hydrogenation of the reduction product gave (−)-menthol predominantly.

This showed that the majority (90%) of isopulegone is reduced to isopulegol by the treatment with sodium in aqueous ammonia, though a minor amount (10%) give menthol through pulegone.

EXPERIMENTAL

All melting and boiling points were uncorrected. Ultra violet spectra were determined at Prof. Shimizu's Laboratory, Shinshu University.

Isopulegone:

Isopulegol, prepared from citronella[10], b.p. 69.0°/6.5 mm, $d_4^{18.5}$ 0.9148, $n_D^{18.5}$ 1.4731, MR 47.31 (Calcd. 47.24), $[\alpha]_D^{18.5}$ $-1.0°$ (homog.), was oxidised by sodium dichromate in acetic acid at 60°. The oxidation product was a colorless oil, which was confirmed to be composed mainly of isopulegone containing a trace amount of pulegone by assay of its ultraviolet spectrum (pure pulegone had λ_{max}^{MeOH} 253 mμ, E_{max}^{MeOH} 7500[11]); Yield 74.1%, b.p. 72.5°/7 mm, d_4^{20} 0.9158, n_D^{20} 1.4696, MR 46.34 (Calcd. 45.72), $[\alpha]_D^{20}$ $-3.7°$ (homog.), λ_{max}^{MeOH} 253 mμ, E_{max}^{MeOH} 137.

Reduction of isopulegone:

Isopulegone (10.0 g) was reduced with sodium (7.5 g) in aqueous ammonia and *n*-hexane (reaction temp. 0∼3°), in the same way as previously reported[1]. The reduction product (9.8 g, Yield 96.7%) was a colorless oil, and did not crystallise on cooling (−22°); b.p. 73∼75°/7 mm, d_4^{20} 0.9028, n_D^{20} 1.4698, MR 47.71 (Calcd. 47.24), $[\alpha]_D^{20}$ $-12.0°$ (homog.), λ_{max}^{MeOH} 268 mμ, E_{max}^{MeOH} 4.

Identification of (−)-isopulegol:

The reduction product (2.07 g) gave crude 3,5-dinitrobenzoate, m.p. 85∼101°, in a quantitative yield. Two crystallisations of its crude ester from *n*-hexane gave pale yellow needles (2.62 g, Yield 56.1% based on the reduction product), m.p. 103∼109°, iodine-number 62.80. The iodine-number was determined by Rosemund-Kuhnhenn's method, using pyridine-bromine-sulphate[5], and it indicated that its 3,5-dinitrobenzoate fraction contained 86.2% of unsaturated ester. One crystallisation from *n*-hexane of the crude bromide (310 mg), recovered from titrated solution of iodine-number determination of its 3,5-dinitrobenzoate (201 mg), gave (−)-dibromo-isopulegyl 3,5-dinitrobenzoate (178 mg, Yield 70.5% for theor.), m.p. 110°, undepressed by admixture with an authentic sample. The authentic (−)-dibromo-isopulegyl 3,5-dinitrobenzoate, m.p. 110°, was prepared from (−)-isopulegyl 3,5-dinitrobenzoate, m.p. 104∼104.5°[10], in the same way as described above.

Catalytic hydrogenation of the reduction product:

The reduction product (3.48 g) was hydrogenated over a platinum oxide catalyst (50 mg) in acetic acid. It absorbed 489 ml (21°C) of hydrogen, equivalent to 89.8% of the theory on the basis of isopulegol, and gave menthol mixture 3.13 g, Yield 88.7% for theor.); b.p. 80∼81°/6 mm, m.p. 22∼23.5°, d_4^{25} 0.8893, n_D^{25} 1.4580, MR 47.94 (Calcd. 47.71), $[\alpha]_D^{25}$ $-29.9°$ (homog.). The menthol mixture (496 mg) gave (−)-menthyl 3,5-dinitrobenzoate (705 mg, Yield 63.4%), m.p. 152∼153°, undepressed

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9) J. L. Simonsen, "The Terpenes" Cambridge Univ. Press. 1953, Vol. I, p. 389.

10) H. Ueda and S. Shimizu, This Bulletin, **24**, 402 (1960).

11) S. Shimizu, *J. Shinshu Univ. Japan*, No. 4, 73 (1954).

by admixture with an authentic sample[12].

Acknowledgements The author is indebted to Emeritus Prof. S. Takei, Prof. T. Mitsui and Prof. M. Nakajima, University of Kyoto, for their kind advices and suggestions. He also wishes to express his sincere thanks to Prof. S. Shimizu, Shinshu University, and Dr. Y. Inouye, Institute for Chemical Research, University of Kyoto, for their kind guidances throughout this series.

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[12] H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 657～666, 1960]

Molecular Complexes of Tetracycline Acid Salts

Part I. The Preparations and Properties of Tetracyclinium Salt-Oxalic Acid Complexes*

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Received May 19, 1960

A series of studies on the crystalline molecular complexes of tetracycline groups have been carried out in this laboratory, and the present paper deals with the compositions of the oxalic acid complexes of various tetracycline acid salts, which were examined by X-ray powder diffraction methods, infrared spectra, and chemical analyses. Insertions of salt anions and crystal water in the crystal lattice of the complexes, and the important role of the latter for crystallization are indicated.

Broad-spectrum antibiotic tetracycline (TC), which was first obtained from catalytic reduction of 7-chlorotetracycline[1] and later by the direct fermentation of a Streptomyces[2], shows interesting physico-chemical behaviors different from other acid and basic antibiotics.

Some properties of TC are attributable to its amphoteric character including unusual insolubility in neutral aqueous solution. Due to these properties, TC hydrochloride is generally used clinically and its alkaline earth metal salts are usually applied for the precipitation of TC from the fermentation broths owing to the sparing solubility of these salts. Aromatic sulfonic acid salts of TC have also been reported[3].

Furthermore, the strong polarity and the polyfunctional character of TC may suggest the formation of complexes having different solubilities. Indeed, many complexes of tetracyclines have been described in the literatures as follows: Calcium chloride complex of 5-

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* S. Inouye and H. Ogawa, The paper presented at the Kanto Local Meeting of the Agricultural Chemical Society of Japan, Feburuary 27, 1960.

[1] J. H. Boothe, J. Morton, J. P. Petisi, R. G. Wikkins and J. H. Williams, *J. Am. Chem. Soc.*, **75**, 4621 (1953); W. T. Moreland, A. R, English, C. R. Stephens and F. J. Pilgrim, *J. Am. Chem. Soc.*, **75**, 4622 (1953).

[2] H. Ogawa and T. Ito, *J. Agr. Chem. Soc. Japan*, **30**, 123 (1956); P. P. Minieri, H. Sokol and M. C. Firman, U. S. Patent No. 2,734,018 (1956).

[3] F. A. Marcketti and G. Casini, *Ricerca. scie.*, **26**, 3321 (1956).

hydroxytetracycline by Regna and co-workers[4], crystalline calcium chloride complex of calcium salt of 7-chlorotetracycline by Ogawa and Ito[5], aluminium α-hydroxycarboxylate complexes of TC and its derivatives by Ritter[6], and TC complexes with benzoic acid and naphthoic acid by Maxion[7], with urea by Smith et al.[8], and with methanol by Söder and Siedel[9]. Sakaguchi et al.[10] indicated in the series of their papers that the enolized 1,3-β-dicarbonyl system in tetracyclines undergoes chelation with various metal ions of 1 : 1 or 1 : 2 type and recently described the mixed complexes of TC-metal chelates[11]. On the other hand, the soluble complexes of TC and 5-hydroxytetracycline were reported by Higuchi et al.[12] However, the above-mentioned complexes were formed chiefly in neutral solutions (pH 3~8) and the complex formation in acid medium has only been reported by Kaplan et al.[13] as hexametaphosphate complex salt.

In the course of isolation and purification of TC from the fermentation broths several years ago, it was observed that TC could easily be crystallized from acid solution by the addition of oxalic acid. A similar complex was also obtained by heating neutral TC with oxalic acid in water or organic solvents containing water.

These complexes are very stable in their solid state, and did not undergo changes in the crystal structure, the antibiotic potencies, or the

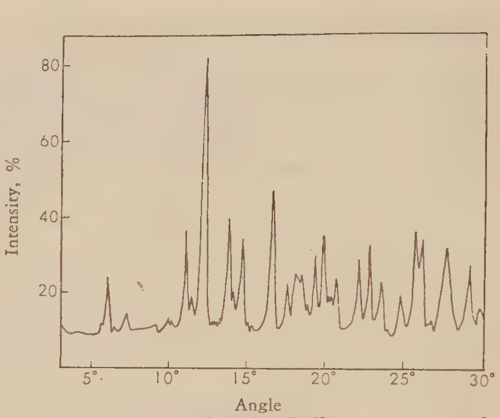

FIG. 1a. X-Ray Powder Diffraction Curve of Tetracyclinium Sulfate-Oxalic Acid Complex.

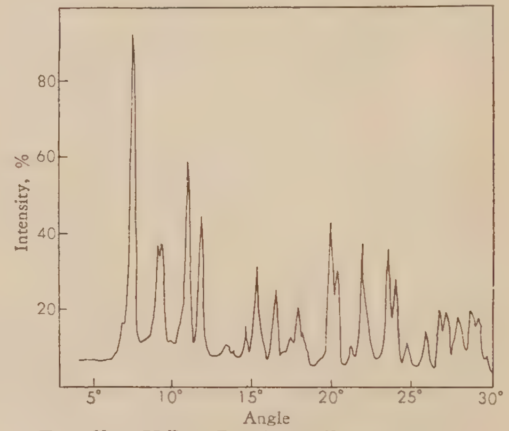

FIG. 1b. X-Ray Powder Diffraction Curve of Tetracyclinium Silicofluorate-Oxalic Acid Complex.

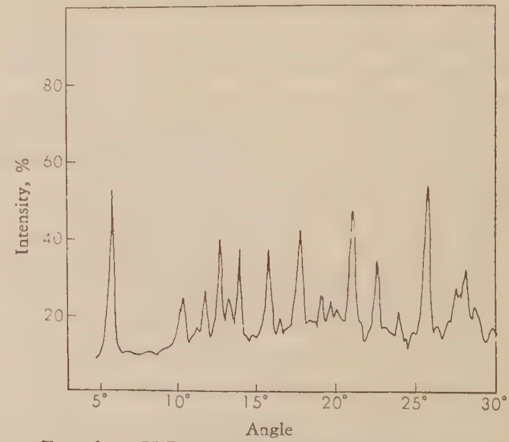

FIG. 1c. X-Ray Powder Diffraction Curve of Tetracyclinium Hemichloride-Oxalic Acid Complex.

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4) P. P. Regna, I. A. Solomons, K. Murai, A. E. Timreck, K. J. Braningo and W. A. Laizier, *J. Am. Chem. Soc.* **73**, 4211 (1951).

5) H. Ogawa and T. Ito, *J. Agr. Chem. Soc. Japan*, **30**, 34 (1956).

6) L. Ritter, U. S. Patent No. 2,736,725 (1956); *Chem. Abstr.*, **50**, 10348 (1956).

7) E. J. Maxion, U. S. Patent No. 2,880,234 (1956); *Chem. Abstr.*, **53**, 10673 (1956).

8) L. L. Smith, S. A. Muller, M. Marx, R. Winterbottom and A. P. Doerschuk, *J. Org. Chem.*, **23**, 721 (1958).

9) A. Söder and W. Siedel, Japanese Patent No. 34-8746 (1959).

10) T. Sakaguchi, M. Toma, T. Yoshida, H. Omura and H. Takasu, *Chem. Pharm. Bull. (Tokyo)*, **6**, 1 (1958) and reference cited there.

11) T. Taguchi, *Chem. Pharm. Bull. (Tokyo)*, **8**, 217 (1960).

12) E. H. Gans and T. Higuchi, *J. Am. Pharm. Assoc.*, **46**, 458 (1957); T. Higuchi and S. Bolton, *ibid.* **48**, 557 (1959).

13) M, A. Kaplan and F. H. Buckmalter, *Antibiotics and Chemotherapy*, **8**, 36 (1958).

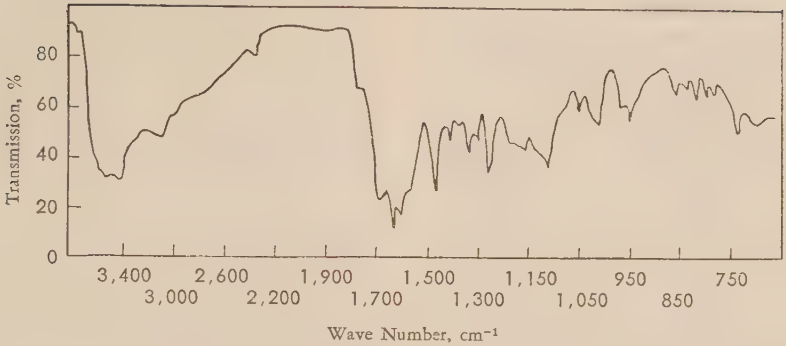

FIG. 2. Infrared Absorption Spectrum of Tetracyclinium Sulfate-Oxalic Acid Complex.

physical appearances by storing at room temperature for three years. The complexes were obtained only from acid solutions and the TC base can be recovered quantitatively from the aqueous solution of such complexes by adjusting the pH to neutral.

Because of the excellent properties of the complexes as mentioned above, including the easy separation from 7-chlorotetracycline the simliar complexes of which do not crystallize out from aqueous solution, they have been conveniently used for the purification of TC[14,15] as follows: The precipitate, which is formed by adjusting the acidified filtrate of fermentation broth of Streptomyces species to pH 8.5 with alkali, is collected and extracted with methanol containing sulfuric acid or oxalic acid. After the methanol solution is evaporated to a small volume in vacuo, TC complex is easily crystallized by the addition of oxalic acid.

From the examination of X-ray powder diffraction patterns, infrared spectra of the complex crystal, and chemical analysis of components, it was found that the TC complex was not a simple oxalate as previously expected[15], but was an oxalic acid complex of TC acid salt. It was also found that oxalic acid complexes prepared in the presence of different strong acids under similar conditions gave varied diffraction patterns indicating the formation of different crystal structures of the complexes (Fig. 1). This result indicates the marked effect of strong acids on the crystal structure of the complexes and suggests the insertion of a strong acid into the complex crystals.

Infrared spectrum of the oxalic acid complex, prepared in the presence of sulfuric acid, showed a strong band at 1108 cm^{-1} for a sulfate an ion and a band at 1730 cm^{-1} for a carbonyl group of undissociated oxalic acid as shown in Fig. 2. It was found by titration and chemical analyses of oxalic acid and sulfur, that this complex consisted of one-half mole of sulfuric acid and one-half mole of oxalic acid, suggesting that TC hemisulfate is combined with one-half mole of the undissociated oxalic acid in the crystal. Furthermore, the strong acidity of oxalic acid was not necessary for the formation of the complex since the neutral compounds, such as oxamide, glyoxal, dicyandiamide, and formamide, formed a similar complex lattice with TC hemisulfate as with oxalic acid[16]. Infrared spectra of oxalic acid complexes of TC oxalate, nitrate, and silicofluorate showed bands at 1550, 1315, and 740 cm^{-1} attributable to the dissociated carboxyl group, nitrate ion, and silicofluorate ion, respectively.

The formation of molecular complexes of TC acid salts, not TC itself, can be attributed, at

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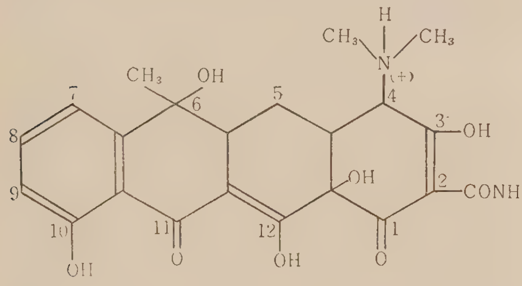
[14] H. Ogawa and S. Inouye, Japanese Patent No. 238,562 (1958).

[15] H. Ogawa and S. Inouye, U. S. Patent No. 2,929,837 (1960).

[16] Detailed experiments of these complexes will be described in a subsequent papar (Part II, *Chem. Pharm. Bull.* (*Tokyo*), in press .

least partly, to the amphoteric character of TC molecule, which has three dissociatable groups showing pKa's values of 3.30, 7.68, and 9.69 in aqueous solution[17]. In a neutral solution, TC probably exists as an inner salt, the enolic hydrogen (pKa 3.30) of tricarbonylmethane group on ring A bonded to the dimethylamino group (pKa 7.68) on C-4, and readily forms insoluble crystal lattice of a zwitter ion even when oxalic acid is co-presented in the solution. In a strong acid solution (below pH 3), it is considered that the dissociation of enolic hydrogen is inhibited and TC exists mainly as a dimethylammonium cation, (tetracyclinium cation) (I), highly solubilizing itself in aqueous solution as in the case of hydrochloride or sulfate, and is incapable of forming an insoluble crystal lattice without the suitable complexing agents.



(I)

The nature of salt anions attached to tetracyclinium cation also shows a marked effect on the physical and chemical properties of the tetracyclinium salt complexes. The effect of various salt anions upon the composition and crystal structure of oxalic acid complexes is rather complicated, because oxalic acid acts both as a salt-and a complex-forming agent. As is shown in Table I, the content of salt anions and of oxalic acid is usually specific in each complex.

TC sulfate-oxalic acid complex can be recrystallized from water containing excess of oxalic acid without a change in the composition. TC silicofluorate-oxalic acid complex also has a similar composition to that of the sulfate complex, but the hydrochloric acid content of TC hydrochloride-oxalic acid complex varies with concentration of the acid present in the solution. If crystallization occurs in water containing excess of hydrochloric and oxalic acids, the complex formed is composed of 1/2 mole of hydrochloric acid and 3/4 mole of oxalic acid, but the complex recrystallized from water containing excess of oxalic acid alone changes in composition to 1/4 mole of hydrochloric acid and one mole of oxalic acid. TC heminitrate and hemihydrobromide form complexes having a similar composition as that of hemihydrochloride, but TC oxalate forms a complex crystal with 3/4 mole of oxalic acid[18].

TABLE I. THE COMPOSITION OF TETRACYCLINIUM SALT-OXALIC ACID COMPLEXES

Complex	Component		
	TC	Strong acid	Oxalic acid
TC sulfate-oxalic acid	1 mole	1/2 mole	1/2 mole
TC hydrochloride-oxalic acid	1	1/2	3/4
TC // - //	1	1/4	1
TC hydrobromide-oxalic acid	1	1/2	3/4
TC nitrate-oxalic acid	1	1/2	3/4
TC oxalate-oxalic acid	1		5/4
TC silicofluorate-oxalic acid	1	1/2	1/2

However, not all the strong acids enter into the complexes. For example, the oxalic acid complex with methanesulfonic acid can not be formed. A complex, crystallized from TC methanesulfonate solution on the addition of oxalic acid, was identified as the TC oxalate-oxalic acid complex by the comparison of X-ray powder diffraction pattern and the elemental analysis. The oxalic acid complex obtained from an aqueous solution of TC phosphate had about the same content of oxalic acid as that of oxalate-oxalic acid complex, but showed a different X-ray powder diffraction pattern.

From the above reactions of various strong acids in the complex formation, order of the preferred insertion of a strong acid into the crystal lattice of oxalic acid complexes would be in the following sequence: H_2SO_4, H_2SiF_6

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[17] C. R. Stephens, K. Murai, K. J. Brunings and R. B. Woodward, *J. Am. Chem. Soc.*, **78**, 4155 (1956).

[18] The special ratio (3/4 : 1) of oxalic acid complex of TC suggests that the complex crystal is not merely the addition compound of each component.

$> (COOH)_2 > HCl$, HNO_3, $HBr > H_3PO_4 \geqq CH_3SO_3H$.

As was mentioned previously, TC sulfate-oxalic acid complex can be well differentiated from TC oxalate complex, hemihydrochloride complex, or from silicofluorate complex by their X-ray powder diffraction patterns, but the differentiation of oxalic acid complexes of TC hemihydrochloride, hemihydrobromide, hemi-nitrate, and 1/4 hydrochloride, was not successful by the same method. The identity of the patterns shown by TC-hydrochloric acid-oxalic acid system is indicative of the isomorphic character of chloride and oxalate ions in these complex crystals.

The melting points (with decomposition) of these various oxalic acid complexes of TC acid salts are unreliable for identification, but are generally lower than those of TC acid salts or its free base.

Each of the tetracyclinium salt-oxalic acid complexes has a specific solubility in water which is summarized in Table II. The comparison of solubilities of TC sulfate-oxalic acid complex and TC hydrochloride in various solvents is shown in Table III, in which the increased solubility of the complexes in ethanol and butanol, and the decreased solubility in water compared to that of hydrochloride are indicated.

The solubility of TC sulfate-oxalic acid complex, however, is markedly influenced by the concentration of the strong acid and the complexing agent. As is illustrated in Fig. 3, the addition of excess sulfuric acid results in a marked decrease in the solubility of the complex and it might be attributable to the inhibition of the dissociation of oxalic acid in the solution.

TABLE II. SOLUBILITIES OF SEVERAL TETRACYCLINIUM SALT-OXALIC ACID COMPLEXES IN WATER AT 0°C

Complex	Solubility (μg/ml)
TC sulfate-oxalic acid	16,000
TC hydrochloride-oxalic acid	22,000
TC hydrobromide-oxalic acid	39,000
TC nitrate-oxalic acid	20,500
TC oxalate-oxalic acid	23,000

TABLE III. COMPARATIVE SOLUBILITIES OF TETRACYLINIUM SULFATE-OXALIC ACID COMPLEX AND TETRACYCLINE HYDROCHLORIDE AT 20°C

Solvent	Tetracyclinium Sulfate-Oxalic acid Complex μg/ml	Tetracycline hydrochloride μg/ml
Water	24,000	74,000
Methanol	110,000	102,000
Ethanol	17,400	6,570
Butanol	1,705	1,070
Acetone	1,180	235
Methyl formate	2,390	1,590
Ethyl acetate	81	41
Chloroform	20	49
Benzene	0	20
Dioxane	2,460	1,230
Formamide	120,500	292,000

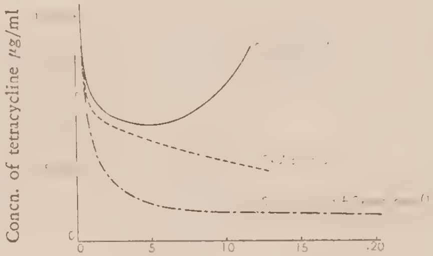

FIG. 3. Phase Diagram Showing the Effect of Sulfuric Acid and Oxalic Acid on the Apparent Solubility of Tetracyclinium Sulfate-Oxalic Acid Complex at 5°C in Water.

The oxalic acid complexes of tetracyclinium salts usually contain crystal water and the presence of water is necessary for the crystallization, because, if anhydrous acetone, ethyl acetate, or butanol is used as the solvent of crystallization, only amorphous complexes are separated, but 2% of water is enough for their crystallization, and the presence of solvated acetone or ethyl acetate is not observed in the infrared spectra. The crystal water plays a definite role in the formation of inter- and intra-molecular hydrogen bondings within the the complexes, since the crystalline complexes do not lose the entire water of hydration on heating at 75~80°C in vacuo for 7 hours.

The ultraviolet absorption spectra of the complexes in aqueous or methanol solution indicate the result of the addition of those of tetracyclinium salt and oxalic acid, and no spectral change relating to the complex formation is observed.

Optical rotations of the complexes in methanol solution are also essentially the same as that of TC hydrochloride based on TC content in the complex molecules.

The interaction of tetracyclinium salt with oxalic acid is further demonstrated by paper chromatography. Paper chromatographic behaviors of complexes with various solvent systems are shown in Table IV. TC sulfate-oxalic acid complex or oxalate-oxalic acid complex can be differentiated from TC hydrochloride using the solvent system (b).

TABLE IV. PAPERCHROMATOGRAPHIC BEHAVIORS* OF TETRACYCLINIUM SALTS AND THEIR COMPLEXES

	Solvent System	
Compounds	(a) *n*-BuOH (4) MeOH (1) H_2O (2.5)	(b) *n*-BuOH (4) AcOH (1) H_2O (2.5)
Tetracycline hydrochloride	0.38	0.64
Tetracyclinium sulfate-oxalic acid complex	0.43	0.57
Tetracyclinium oxalate-oxalic acid complex	0.45	0.52

* Filter paper, Toyô Roshi No. 50; Ascending; Indicator, Ultraviolet Lamp.

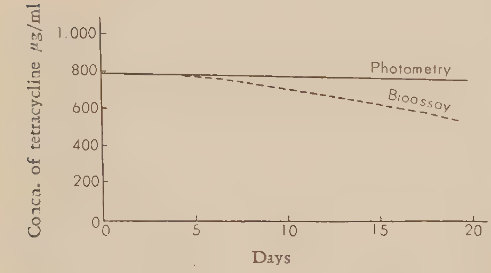

FIG. 4. Stability of Tetracyclinium Sulfate-Oxalic Acid Complex in Aqueous Solution at 20°C.

The stability of TC sulfate-oxalic acid complex in aqueous solution is shown in Fig. 4, and it suggests that little alteration of TC occurs, in the solution either by anhydro-formation[19] at C-6 or epimerization at C-3[20].

EXPERIMENTAL

Spectrophotometric assays were made in 0.1N sulfuric acid solution, using EPU-2 quartz spectrophotometer, and microbiological potencies were assayed by the cylinder plate method using *Bacillus subtilis* PCI 219, and they were compared with the pure standard tetracycline (TC) hydrochloride, 1,000 μg/mg.

Infrared spectra were measured as a KBr tablet with Kōken DS-301 spectrometer.

X-Ray Apparatus and Technique

Geigerflex diffractometer, with a Geiger-counter tube, was used for recording the X-ray diffraction patterns. Copper K_α radiation (wave length: $\lambda=1.5418$ Å) was obtained from a target tube, filtered through a nickel foil, operated at a potential of 35 KV and a current of 20 mA (or 15 mA) with time constant of 2 seconds, goniometer velocity of 2°/min., and recording speed of 2 cm/min.

Compounds were carefully powdered and patterns were recorded using the powder slab technique. X-Ray powder diffraction data showed the interplaner spacings in Å along with relative intensity, which is recorded relative to the intensity of the highest peak (designated as 1.00) of each pattern. The low d spacings at about 2.98 Å and the weak peaks of diffraction were cut off.

Determination of Acids Contained in the Complex

Accurately weighed tetracyclinium salt-oxalic acid complex (about 10 mg) was dissolved in 15 ml of water and titrated with 0.01N sodium hydroxide solution, standardized against pure oxalic acid dihydrate, with methyl red as an indicator. Oxalic acid and hydrochloric acid could be titrated quantitatively in the presence of TC as well as the hydrochloric acid in TC-HCl by the same condition.

Determination of Oxalic Acid in the Complex

Accurately weighed tetracyclinium salt-oxalic acid complex (about 50 mg) was dissolved in 6 ml of water and 50% sulfuric acid (0.15 ml) and passed through a column of Amberlite IR-120 (H^+ form) (10 ml) packed in a glass tube of 1 cm in diameter and previously washed with water (20 ml) and 50% sulfuric acid (0.1 ml)[21]. The column was washed with a solution of 50% sulfuric acid (0.1 ml) in 10 ml of water and then with

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[19] S. Inouye, T. Ito and H. Ogawa, *J. Agr. Chem. Soc. Japan*, **30**, 591 (1956).

[20] J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitter, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 2849 (1957).

[21] It was necessary to maintain strong acidity in order to obtain quantitative absorption of TC to the cation exchange resin owing to the amphoteric character of TC.

15 ml of water. To the combined acid effluent was added one ml of 50% sulfuric acid. After the solution was ascertained to show no absorbance of TC at 355 mμ, the solution was titrated with 0.05M potassium permanganate at 60°C. The hydrochloric acid or nitric acid contained in the complex did not interfere in the titration with permanganate.

Tetracyclinium Sulfate-oxalic Acid Complex

A solution of TC (500 mg) dissolved in 5 ml of water with 50% sulfuric acid (0.2 ml) was filtered and agitated with finely powdered oxalic acid dihydrate (250 mg) and the immediately separated crystals were collected by filtration after cooling overnight, washed with cold water, and dried in vacuo over potassium hydroxide. It weighed 460 mg; oxalic acid content: 7.4%. X-Ray powder diffraction pattern is shown in Fig. 1 and has the following data.

X-Ray powder diffraction data

d, Å	I/I$_1$	d, Å	I/I$_1$
14.49	0.30	4.46	0.43
7.97	0.44	4.29	0.30
7.25	1.00	4.04	0.36
6.42	0.49	3.90	0.41
6.03	0.43	3.77	0.30
5.34	0.58	3.48	0.44
5.01	0.28	3.41	0.42
4.87	0.31	3.23	0.40
4.77	0.31	3.08	0.35
4.58	0.37		

The above complex, (500 mg) was slurried in water with oxalic acid dihydrate (100 mg) and heated at 70°C until in complete solution, filtered, and cooled immediately. After 5 hr. in refrigerator, the deposited crystals were collected and dried in vacuo at room temperature. It weighed 291 mg, assayed 840 μg/mg (spectrophotometric) and 890 μg/mg (microbiological), decomposed at 196~198°C with sintering from 156°C, $[\alpha]_D^{20}$: −216°C (c, 0.4165 in methanol).

Ultraviolet spectra of the tetracyclinium sulfate-oxalic acid complex in 0.1N and 0.01N sulfuric acid exhibited maxima at 217, 271, and 355 mμ, and minima at 233 and 300mμ. TC–HCl also had three maxima at the same wave lengths.

Infrared spectrum of tetracyclinium sulfate-oxalic acid complex as a potassium bromide disk is shown in Fig. 2, indicating bands at 3440, 3340, 3030, 1665, 1615, 1590, 1545, 1450, 1400, 1370, 1320, 1290, 1255, 1240, and 1135 cm^{-1}, attributable to tetracyclinium cation, at 1730 cm^{-1} to oxalic acid, at 1108 cm^{-1} to sulfate ion, and at 1155 and 1020 cm^{-1} not assigned.

No variation was observed in X-ray powder diffraction pattern by recrystallization of the complex. The water content in the complex was determined by direct titration with the Karl-Fisher reagent, using methanol as a solvent.

Found: C, 48.3; H, 5.3; N, 5.15; acids, 2.0 eq.; oxalic acid, 8.4%; H_2O, 7.3%.

Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2H_2SO_4 \cdot 1/2C_2H_2O_4 \cdot 2H_2O$: C, 48.1; H, 5.3; N, 4.9; S, 2.8; acids, 2.0 eq., oxalic acid, 7.9%; H_2O, 6.3%.

The analytical values of the sample heated at 75°C for 7 hr. in vacuo over phosphorus pentoxide were: C, 48.3; H, 5.45; N, 4.4; S, 3.8; acids, 2.1 eq.; H_2O, 4.7%. No alteration of X-ray powder diffraction pattern was detected by heating the sample.

Conversion of tetracyclinium sulfate-oxalic acid complex to TC base was as follows: 2.0 g of tetracyclinium sulfate-oxalic acid dihydrate was dissolved in 50 ml of warm water, and the solution was filtered. Neutralization with 3 ml of 10% sodium hydroxide caused immediate crystallization and, after standing in a refrigerator overnight, the crystals were collected and dried over phosphorus pentoxide in vacuo. It weighed 1.6 g (95% yield), assayed 950 μg/mg (microbiological), and was determined as monohydrated TC by the X-ray powder diffraction pattern.

X-Ray powder diffraction data

d, Å	I/I$_1$	d, Å	I/I$_1$
9.83	0.96	4.58	0.34
9.31	0.51	4.44	0.44
8.42	0.39	4.21	0.66
7.56	1.00	3.95	0.41
6.81	0.39	3.69	0.31
6.71	0.35	3.58	0.43
5.83	0.40	3.41	0.30
5.01	0.70		
4.62	0.34		

Found: C, 56.9; H, 5.7; N, 5.8; H_2O, 5.1%.

Calcd. for $C_{22}H_{24}N_2O_8 \cdot H_2O$: C, 57.2; H, 5.7; N, 6.1: H_2O, 3.9%.

For comparison, TC–HCl was prepared in usual way by dissolving 2 g of TC monohydrate in 16 ml of butanol and conc. hydrochloric acid (0.5 ml), and stirring the solution for 60 hrs. at room temperature with further addition of hydrochloric acid (0.3 ml). The precipitated crystals were collected to yield 1.6 g of TC–HCl, showing the same assay value with the standard sample (1000 μg/mg).

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
10.16	0.38	4.48	0.62
9.12	0.41	4.29	0.54
8.04	0.68	4.17	0.44
7.50	0.44	4.08	0.51
6.46	0.63	3.83	0.44
5.79	0.59	3.71	0.46
5.61	1.00	3.68	0.49
5.28	0.50	3.12	0.56
4.85	0.53		
4.55	0.47		

Tetracyclinium Oxalate-Oxalic Acid Complex

A. Preparation from the aqueous solution

Hydrated TC (1 g, 900 μg/mg) dissolved in 8 ml of water with triethylamine (0.5 ml) at 40°C, was filtered immediately. The filtrate was acidified with oxalic acid dihydrate (1 g) and cooled for 3 hrs., recovering TC complex (1 g). Recrystallization from warm water (10 ml) containing oxalic acid dihydrate (100 mg) gave the complex (752 mg). Potency 850 μg/mg, d.p. ca. 140°C sintering at 112~122°C. $[\alpha]_D^{20}$: −214° (c, 0.4485 in methanol).

U.V. (0.1N sulfuric acid): λ_{max} 213, 270, and 355 mμ.

I.R. (KBr) (characteristic bands differed from that of TC-HCl); 1730 (COOH), 1565 (COO^-), 1340 (COO^-), 1100 (not assigned), and 1070 cm^{-1} (not assigned).

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
15.24	1.00	4.23	0.48
7.63	0.46	3.48	0.60
6.86	0.35	3.43	0.38
6.28	0.35	3.24	0.29
5.04	0.43		

Found: C, 49.8; H, 5.0; N, 4.5; acids, 2.6 eq. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 5/4C_2H_2O_4 \cdot 2H_2O$: C, 49.6; H, 5.2; N, 4.7; acids. 2.5 eq.

The sample was heated at 75°C for 7 hrs. in vacuo over phosphorus pentoxide.

Found: C, 51.8; H, 5.1; N, 4.6; acids, 2.7 eq.; oxalic acid, 20.9%, 20.3%. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 5/4C_2H_2O_4 \cdot H_2O$: C, 51.2; H, 5.0; N, 4.9; acids, 2.5 eq.; oxalic acid, 19.6%.

The same complex was obtained more readily by dissolving TC-hydrate (1 g) in water (10 ml) with oxalic acid dihydrate (500 mg), warmed at 70°C until in complete solution, and cooling for 4 hrs. (yield, 63%).

B. Preparation from methanesulfonate solution

TC-hydrate (1 g) was dissolved in water (5 ml) with methanesulfonic acid (0.2 ml), oxalic acid dihydrate (500 mg) was added to the solution, and the resulting crystals (700 mg) were recrystallized from hot water (7 ml) with methanesulfonic acid (0.03 ml) and oxalic acid dihydrate (50 mg). Yield 380 mg. The product was identified as tetracyclinium oxalate-oxalic acid complex by the X-ray powder diffraction pattern and by analysis of sulfur (0.48%).

C. Preparation from butanol solution

Anhydrous TC (300 mg), prepared by drying at 80°C in vacuo, was dissolved in 30 ml of butanol containing anhydrous oxalic acid (200 mg) at 80°C and the solution was chilled (4°C) to deposit an amorphous complex (100 mg, 800 μg/mg, microbiological), which consumed 1.6 moles of alkali per mole of TC corresponding to $C_{22}H_{24}N_2O_8 \cdot 4/5C_2H_2O_4$, acids, 1.6 eq.

On the other hand, in the same condition as mentioned above, 160 mg of crystalline oxalic acid complex was obtained by the addition of only 0.5 ml of water to the solution. X-Ray powder diffraction pattern of the crystalline complex was essentially the same with that from aqueous solution and also from hydrous acetone or ethyl acetate.

Tetracyclinium Chloride-Oxalic Acid Complex

TC-HCl (1 g) was dissolved in water (8 ml), oxalic acid dihydrate (500 mg) and conc. hydrochloric acid (0.3 ml) were added, and after cooling for 2 hrs., the crystals were collected and dried in vacuo. It weighed 1.70 g. They were recrystallized from water (9 ml) with oxalic acid dihydrate (50 mg) and conc. hydrochloric acid (0.1 ml), yielding 1.3 g of a sample which melted with decomposition at 148~150°C. Potencies: 800 μg/mg (spectrophotometric) and 850 μg/mg (microbiological).

X-Ray powder diffraction curve is shown in Fig. 1 and has following data.

d, Å	I/I_1	d, Å	I/I_1
14.98	0.99	4.51	0.46
8.51	0.46	4.42	0.41
7.50	0.50	4.23	0.87
6.97	0.74	3.93	0.65
6.66	0.46	3.72	0.41
6.33	0.70	3.47	1.00
5.61	0.70	3.24	0.52
5.01	0.80	3.18	0.61
4.65	0.48	3.11	0.43

Found: C, 50.1; H, 5.5; N, 4.8; acids 2.1 eq.; H_2O, 6.9%. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HCl \cdot 3/4C_2H_2O_4 \cdot 2H_2O$; C, 49.9; H, 5.3; N, 5.0; acids, 2.0 eq.; H_2O, 6.4%.

The sample dried at 75°C for 7 hrs. in vacuo over phosphorous pentoxide was analyzed.

Found: C, 51.9; H, 5.0; N, 4.6; Cl, 2.9; acids, 1.9 eq. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HCl \cdot 3/4C_2H_2O_4 \cdot H_2O$,: C, 51.5; H, 5.15; N, 5.1; Cl, 3.2; acids, 2.0 eq.

The above complex (500 mg) was recrystallized from 6 ml of water with oxalic acid dihydrate (50 mg), yielding 300 mg; assayed 840 μg/mg (microbiological). X-Ray powder diffraction pattern was essentially the same as that of the complex of tetracyclinium hemichloride, with some difference in intensity.

For analysis the sample was dried in vacuo at 75°C for 7 hrs. over phosphorous pentoxide. Found: C, 51.3; H, 5.4; N, 4.6; Cl, 1.8; acids, 2.24 (or 2.20) eq., oxalic acid, 15.1%. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/4HCl \cdot C_2H_2O_4 \cdot 3H_2O$: C, 52.0; H, 5.8; N, 5.1; Cl, 1.6; acids, 2.25 eq., oxalic acid, 16.2%. (Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/4HCl \cdot 7/8C_2H_2O_4 \cdot H_2O$: C, 51.2; H, 5.2; N, 5.1; Cl, 1.6; acids, 2.0 eq., oxalic acid, 14.5%.)

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
15.24	1.00	4.77	0.27
8.67	0.30	4.53	0.29
8.51	0.33	4.35	0.27
7.50	0.43	4.23	0.60
6.97	0.49	3.93	0.37
6.66	0.34	3.45	0.59
6.33	0.38	3.22	0.31
5.61	0.68	3.16	0.40
4.98	0.57	3.12	0.29

Tetracyclinium Nitrate-Oxalic Acid Complex

Tetracyclinium nitrate-oxalic acid complex (469 mg) obtained by dissolving TC (500 mg) in 2.5 ml of water with the addition of conc. nitric acid (0.1 ml) and oxalic acid dihydrate (250 mg), melted at 129∼134°C with decomposition and showed the same infrared spectrum as those of the corresponding chloride or bromide complex except the strong bands at 1337 cm^{-1} and 1315 cm^{-1} which were attributed to the nitrate ion.

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
15.00	0.87	4.58	0.43
8.51	0.47	4.46	0.39
7.56	0.77	4.23	0.13
6.92	0.74	3.93	0.65
6.71	0.58	3.72	0.46
6.33	0.68	3.49	1.00
5.64	0.58	3.28	0.46
5.44	0.39	3.20	0.55
5.01	0.85	3.13	0.45
4.62	0.47		

Found: C, 48.8; H, 5.2; N, 6.3: Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HNO_3 \cdot 3/4C_2H_2O_4 \cdot 2H_2O$: C, 48.7; H, 5.2; N, 6.0.

The sample was heated at 75°C for 7 hrs. in vacuo over phosphorous pentoxide.

Found: C, 49.4; H, 5.3; N, 6.3; acids, 1.9 eq.

Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HNO_3 \cdot 3/4C_2H_2O_4 \cdot 3/2H_2O$: C, 49.5; H, 5.1; N. 6.1; acids, 2.0 eq.

Tetracyclinium Bromide-oxalic Acid Complex

Tetracyclinium bromide-oxalic acid complex (785 mg) was prepared by the similar method as the nitrate complex, by dissolving TC (1 g) in 7.5 ml of water with 40% hydrobromic acid (0.35 ml) and oxalic acid dihydrate (500 mg). Recrystallization of this complex (500 mg) from 4 ml of water with 40% hydrobromic acid (0.01 ml) and oxalic acid dihydrate (50 mg) gave 308 mg of the complex, m.p. 155∼160°C (decomp.)

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
15.20	0.81	6.37	0.60
8.51	0.39	5.64	0.53
7.56	0.44	5.37	0.36
7.03	0.53	5.01	0.72
6.71	0.45	4.82	0.33
4.65	0.41	3.72	0.40
4.55	0.37	3.47	1.00
4.44	0.40	3.25	0.44
4.23	0.68	3.18	0.59
3.95	0.57	3.12	0.38

Found: C, 49.2; H, 5.3; N, 4.9; acids, 2.0 eq.; H_2O, 5.7%. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HBr \cdot 3/4C_2H_2O_4 \cdot 2H_2O$: C, 48.0; H, 5.1; N, 4.8; acids, 2.0 eq.; H_2O, 5.8%.

A sample was dried at 75°C for 7 hrs. in vacuo. Found: C, 50.9; H, 5.0; N,4.4; acids, 1.9 eq. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2HBr \cdot 3/4C_2H_2O_4$: C, 51.1; H, 4.75; N, 5.1; acids, 2.0 eq.

Tetracyclinium Silicofluorate-Oxalic Acid Complex

Tetracyclinium silicofluorate-oxalic acid complex (950 mg) was prepared by the similar method as above, by dissolving TC hydrate (1 g) in 10 ml of water with dilute hydrosilicofluoric acid (0.5 ml) and oxalic acid dihydrate (500 mg). m.p. 102∼105°C.

Infrared spectrum showed a strong band at 740 cm^{-1} assigned to Si–F bond in SiF_6^{-2}.

X-Ray powder diffraction curve is shown in Fig. 1 and has the following data.

d, Å	I/I_1	d, Å	I/I_1
12.63	0.18	4.35	0.32
11.48	1.00	4.04	0.42
9.61	0.40	3.99	0.18
9.35	0.41	3.77	0.40
7.97	0.64	3.71	0.30
7.38	0.48	3.44	0.16
6.07	0.16	3.33	0.22
5.75	0.34	3.28	0.20
5.37	0.28	3.19	0.20
4.96	0.22	3.11	0.22
4.44	0.47	3.06	0.20

Found: C, 43.8; H, 5.7; N, 4.5; H_2O, 10.9%. Calcd. for $C_{22}H_{24}N_2O_8 \cdot 1/2H_2SiF_6 \cdot 1/2C_2H_2O_4 \cdot 4H_2O$: C, 43.7; H, 5.4; N, 4.4; H_2O, 11.4%.

Oxalic Acid Complex Obtained from Tetracyclinium Phosphate

A complex (150 mg) was obtained from cold aqueous solution (10 ml) of TC (600 mg), conc. phosphoric acid (0.2 ml), and oxalic acid dihydrate (250 mg). Oxalic acid content: 18.5%.

X-Ray powder diffraction data

d, Å	I/I_1	d, Å	I/I_1
16.99	0.83	4.44	0.54
8.04	0.70	4.23	0.52
7.76	0.54	4.04	0.50
7.14	0.65	3.83	0.59
6.37	0.78	3.51	0.89
5.25	0.74	3.39	0.70
5.07	0.54	3.21	0.57
4.82	0.53	3.18	0.65
4.55	1.00	3.08	0.54

Isolation of TC as Tetracyclinium Salt-Oxalic Acid Complex from Fermentation Broths

A. As tetracyclinium sulfate-oxalic acid complex

Seven liters of a fermentation broth of Streptomyces containing TC as the main antibiotic were acidified with 50% sulfuric acid to pH 1.8 and filtered with aid of Dicalite. To the filtrate a 50% aqueous solution of Cation F_2–50[22] (17.5 ml) was added and the pH was adjusted to 8.5 with 10% sodium hydroxide solution. After standing at room temperature overnight, the precipitate was collected by filtration, washed with water, and dried at 40°C for 8 hrs., yielding 47 g of fine powdered cake.

The cake (50 g), having 300 μg/mg of antibiotic activity, was slurried with methanol (400 ml) and 50% sulfuric acid (15 ml) to bring the pH to 1.7, and stirred for 2 hrs. at room temperature and filtered. The cake was extracted twice more with methanol and sulfuric acid (150 ml each) at pH 1.8 and filtered. The combined acid extracts were treated with 15 g of activated carbon (Darco-G-60) for 2 hrs., and filtered after adjusting to pH 3.0 with 10% sodium hydroxide solution (16 ml). The filtrate was concentrated at a reduced pressure below 35°C. To the concentrate (28 ml), having the potency of 100,000 μg/ml, oxalic acid dihydrate (2.5 g) was added and crystals that separated immediately after oxalic acid dissolved were collected by cooling overnight, washed with cold water, and dried in vacuo at room temperature, weighed, 4.714 g: bioassayed, 850 μg/mg.

B. As tetracyclinium oxalate-oxalic acid complex

The dried cake (60 g) obtained as above, having a total potency of 16,800 mg, was extracted three times with methanol (1 l) and oxalic acid dihydrate (39 g). The extracts were decolorized with activated carbon (15 g) and concentrated to dryness at reduced pressure below 35°C. The residue was dissolved in isoamyl alcohol (15 ml) with warming and the crystals, separated by standing, were collected by filtration after cooling overnight, and dried in vacuo at 50°C; weighed 11.85 g (50% yield from methanol extract); bioassayed, 710 μg/mg.

Determination of the Solubility

A portion of a TC complex was suspended in a solvent and stirred for one hr. at 0°C or at room temperature, and the filtrate was assayed spectrophotometrically.

Determination of the solubility of tetracyclinium sulfate-oxalic acid complex in the presence of oxalic acid and excess sulfuric acid was done as follows: Accurately weighed tetracyclinium sulfate-oxalic acid dihydrate and oxalic acid dihydrate were dissolved with warming in water containing certain moles of sulfuric acid and the solution was cooled to 4°C. After standing for 48 hrs., with frequent agitation, the deposited crystals were filtered off and the filtrate was analyzed spectrophotometrically. During these experiments, the possible formation of anhydro-TC due to a rather strong acidic condition was checked, but no evidence was observed by ultraviolet spectrum.

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[22] Cation F_2-50 is essentially lauryl trimethylammonium chloride; obtained from Nippon Yushi Co., Japan.

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Studies on the Constituents of Hemp Plant (*Cannabis sativa* L.)

Part I. Volatile Phenol Fraction

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Examination of the volatile compounds from Japan hemp plant shows that the phenol fraction consists mainly of eugenol with small amounts of guajacol. Two unknown carbonyl compounds are separated as their 2,4-dinitrophenylhydrazones by the silicic acid-celite column chromatography from the phenol fraction.

INTRODUCTION

As the volatile constituents of hemp resin, a terpene b.p. 165~175°C, possibly myrcene, and a sesquiterpene were reported by Wood, Spivey, and Easterfield1). In 1942, Simonsen and Todd2) examined the essential oil from Egyptian hashish of uncertain age, and showed that the "lower-boiling terpene fraction" consists mainly of *p*-cymene with small amounts of 1-methyl-4-isopropenyl-benzene and unidentified optically active material. From the "higher-boiling fraction" of the oil, humulene ($\underline{\alpha}$-caryophyllene) was isolated.

In the course of our investigations on hemp plant constituents, the authors examined the volatile phenol fraction and identified eugenol and a trace of guajacol by paper chromatography. After purification of 3,5-dinitrobenzoate derived from the phenol fraction by silicic acid-celite column chromatography, mixed melting points, elemental analysis and infrared spectra proved it as eugenol 3,5-dinitrobenzoate. Eugenol has never been isolated from Moraceae family.

From the aqueous solution of 20% sodium bisulfite which was used to separate the carbonyls from the crude phenol fraction, two unidentified 2,4-dinitrophenylhydrazones, m.p. 210°C and 105~107°C were obtained.

EXPERIMENTAL

Sample:

Minamioshihara No. 1 variety of the domestic hemp, raised in the experimental field of Hokkaido University during the season of 1958 was used in this study. It was cut in August just before efflorescence. About 100 kg of hemp leaves and tops were gathered immediately and extracted with petroleum ether (b.p. 30~50°C) at the room temperature. Extraction was repeated and the total number of 210 l of petroleum ether was used. Most of the solvent was recovered under 70°C of water bath and 22.2 g of the solids was separated by centrifuge.

The supernatant, about 2 l, was shaken with 5% sodium bicarbonate. After combining the petroleum ether layer with the washings of the precipitate which was formed by the shaking with bicarbonate, 5 l of the solution was shaken with aqueous 5% sodium hydroxide solution. The aqueous extract, acidified with sulfuric acid and saturated with sodium chloride, was shaken with ethyl ether repeatedly and thus crude phenols were obtained. Yield 4.9 g, red oil. The crude phenols were steam-distilled, and the distillate of an amount of 2.5 l was saturated with sodium chloride and extracted with ether repeatedly. The volatile phenol fraction, red oil, was obtained from the ether solution. Yield 1.4 g.

Detection of Phenols by Paper Chromatography:

Detetion of phenols in the volatile phenol fraction was carried out by paper chromatography, preparing

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1) T. B. Wood, W. T. N. Spivey and T. H. Easterfield, *J. Chem. Soc.*, **69**, 539 (1896).

2) J. L. Simonsen and A. R. Todd, *J. Chem. Soc.*, **1942**, 188.

their oxyazocompounds according to the methods of Hossfeld et al.[3,4]. Two drops of the crude phenol fraction was dissolved in 0.1 ml of aqueous 8% sodium hydroxide. Three drops of diazotized sulfanilic acid were added in this solution. After being acidified with an excess of concentrated hydrochloric acid, the reaction mixture was extracted once with benzene. The aqueous solution was diluted with alcohol and spotted on the position at 4 cm from the below end of the filter paper which had been sprayed with aqueous 4% sodium carbonate and dried. Water saturated *n*-butanol, a mixture of *n*-butanol add 28% ammonia water (4 : 1, v/v) or *sec*-butanol saturated with aqueous 2% sodium carbonate were used for irrigating solvent and developed upward at the room temperature. Eugenol and traces of guajacol and few other phenolic compounds were detected in each solvent system.

Isolation and Identification of Eugenol 3,5-Dinitrobenzoate:

The crude phenol fraction was dissolved in 50 ml of ether and shaken with an aqueous solution of 20% sodium bisulfite to separate the carbonyls. After removal of the carbonyl compounds and being dried, about 1 g of red oil was dissolved in 3 ml of anhydrous pyridine and 0.5 g of 3,5-dinitrobenzoyl chloride was added. The mixture was warmed over a low flame for a minute and poured into 10 ml of water. The light brown precipitate was separated and stirred thoroughly with 20 ml of 2% sodium bicarbonate solution, and recrystallized from ethyl alcohol. One hundred and sixty mg of slightly colored needles m.p. 130°C was obtained.

The 3,5-dinitrobenzoate was dissolved in a small amount of benzene and added on the top of the column consisting with silicic acid and celite mixed at the rate of 2 : 1 in weight. The column, 10 cm in its height, was eluted with a mixture of petroleum ether and ethyl ether (5% by volume). Total eluate from the column was 800 ml. The solvent was evaporated and the residue was recrystallized from ethyl alcohol. White crystals, 57 mg, m.p. 130～131°C. An additional crops of 41 mg, m.p. 130～131°C, was obtained from the filtrate. *Anal.* Found: C, 57.0; H, 4.0; N, 7.58. Calcd. for $C_{17}H_{14}O_7N_2$: C, 57.0; H, 3.9; N, 7.82.

The infrared spectrum of this crystal was identical with 3,5-dinitrobenzoate of the authentic eugenol. The mixed melting point of these derivatives did not depress.

Carbonyls is Crude Phenol Fraction:

The aqueous 20% sodium bisulfite solution which was used to separate the carbonyls from the volatile phenol fraction was washed with ether, added with 6N sulfuric acid and extracted repeatedly with ether. After distilling off the ether, about 400 mg of red oil was obtained. This oil was poured into the solution consisting of 0.5 g 2,4-dinitrophenylhydrazine, 2 ml concentrated sulfuric acid, 3 ml water and 40 ml ethyl alcohol. After a few days, a separated reddish precipitate of 2,4-dinitrophenylhydrazone, 160 mg, was filtered off and dried over P_2O_5.

Column Chromatographic Separation of 2,4-dinitrophenylhydrazones:

A mixture of silicic acid and celite, at the rate of 2 : 1 in weight, was added with petroleum ether into a chromatographic glass tube to build a column (27×250 mm). A chloroform solution of 20 mg 2,4-dinitrophenylhydrazones was poured onto the column. After absorption of the chloroform solution, 200 ml of petroleum ether containing ethyl ether (2% by volume) was added on the column. As separation of the bands occurred, the ethyl ether content of the mixture solvent was increased to 5% and 10% (by volume) and eluted 300 ml and 750 ml respectively. After the first yellow zone (Band No. 1) had been eluted out, 4 bands (No. 2～5) were extruded, sectioned and eluted with ethyl ether. The result obtained with three repetition are shown in Table I.

TABLE I. CHROMATOGRAPHIC SEPARATION OF 2,4-DINITROPHENYLHYDRAZONES OBTAINED FROM THE VOLATILE PHENOL FRACTION OF HEMP PLANT

Band No.	Color of Band	Yield (mg)
1	Yellow	37
2	Orange	20
3	Orange	6
4	Yellow	
5	Orange brown	4

Band No. 1:

Two successive recrystallization from a mixture of petroleum ether and ethyl ether gave yellow crystals, m.p. 105～107°C, 20 mg. The infrared spectrum of this crystal, shown in Fig. 1, was compared with those of authentic 2,4-dinitrophenylhydrazones of *n*-heptylic aldehyde (m.p. 105～106°C) and *n*-valeric aldehyde (m.p. 107～108°C), however, did not agree with each one of them.

Band No. 2:

The 2,4-dinitrophenylhydrazone, remained in the flask after removing of the solvent used to elute the orange band (No. 2), was recrystallized from a mixture of ethyl

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[3] R. L. Hossfeld, *J. Am. Chem. Soc.*, **73**, 852 (1951).

[4] Wen-Hua Chang, R. L. Hossfeld and Wm. M. Sandstrom, *J. Am. Chem. Soc.*, **74**, 5766 (1952).

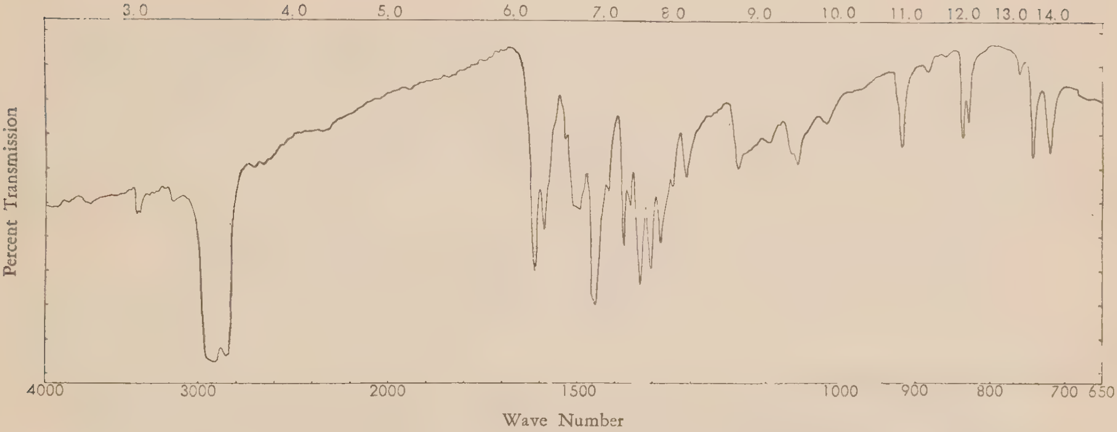

FIG. 1. Infrared Absorption Spectrum of Unknown 2,4-Dinitrophenylhydrazone (m.p. 105~107°C, Band No. 1). Obtained from Hemp Plant (Nujol).

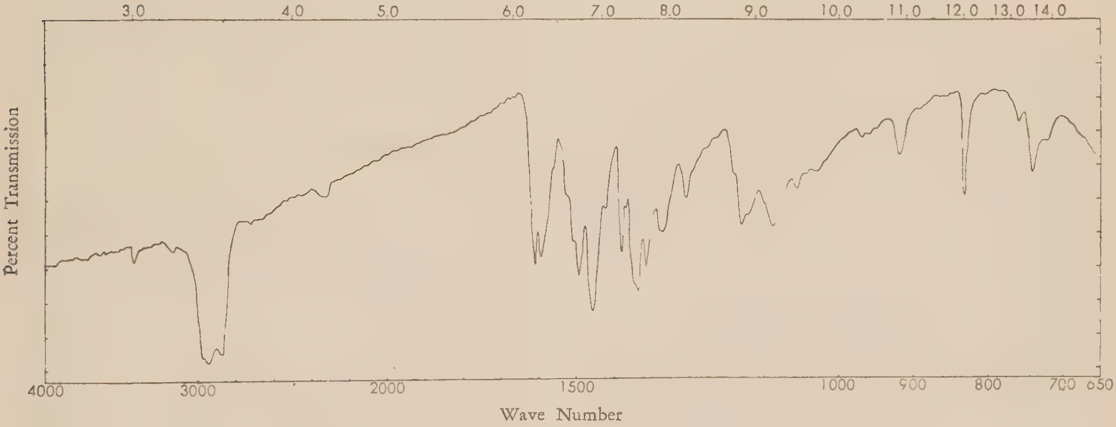

FIG. 2. Infrared Absorption Spectrum of Unknown 2,4-Dinitrophenylhydrazone (m.p. 210°C, Band No. 2). Obtained from Hemp Plant (Nujol).

ether and chloroform and the melting point was raised to 210°C. The infrared spectrum of this red crystal, shown in Fig. 2, did not agree with authentic 2,4-dinitrophenylhydrazone of *o*-hydroxacetophenone (m.p. 210~213°C) or 5-methyl furfural (m.p. 213~214°C) prepared from sucrose according to the method of Scott and Johnson[5].

We could not identify any component of the carbonyls because of the lack of sufficient amount for further investigation.

Acknowledgement The authors wish to express their thanks to Mr. K. Narita and Mr. T. Oishi, Department of Pharmacology, Hokkaido University for the microanalysis and the infrared analysis. They are also greatly indebted to Mr. S. Hatae, T. Hasegawa Co., Ltd. for kindly supplying *n*-heptylic aldehyde.

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[5] E. W. Scott and J. R. Johnson, *J. Am. Chem. Soc.*, **54**, 2549 (1932).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 670～672, 1960]

Studies on the Components of the Hemp Plant (*Cannabis sativa* L.)

Part II. Isolation and Identification of piperidine and Several Amino Acids in the Hemp Plant

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Received June 4, 1960

Investigation was undertaken to ascertain the components of the unpleasant odor of the hemp plant. The authors isolated and identified piperidine, as one of the unpleasant odor components of the plant, in ethanol soluble fraction of the plant.

INTRODUCTION

Almost all species of wild hemp exude a strong unpleasant odor in its flowering time and a lot of flies can be seen flying around the flower during this time.

Wild hemp used in this experiment was gathered right after the plant had flowered and was investigated for its unpleasant odor components.

In the previous paper the authors indicated that during the exploratory experiment on sulphur compounds in the process of beer brewing, humulone and lupulone in hop were found to be the cause of sunlight flavor of beer.[1~6] In this work the authors investigated the sulphur compounds in wild hemp to be helpful for elucidating the mechanism of sunlight flavor of beer because of hemp (*Cannabis sativa* L.) which in the same family (*Moracea*) with hop gives the similar unpleasant odor to the sunlight flavor of beer. Up to the present, free piperidine, combined piperidine and the following amino acids, leucine (or isoleucine), valine, methionine, threonine, histidine, arginine and lysine were isolated and identified from ethanol soluble fraction of the wild hemp.

EXPERIMENTAL AND RESULTS

Sample:

Hemp used in this experiment was one that grew wild in the fields of YUBARI COUNTY in HOKKAIDO.

Isolation and Identification of Piperidine by Paper Chromatography:

Leaves and tops of wild hemp (10 kg) were extracted with petroleum ether (56 l) (b.p. 28～42°C) four times at room temperature and then the residue was extracted with 90% ethanol (50 l) three times at room temperature, this ethanol soluble fraction was concentrated in reduced pressure.

In the alcohol distillate, piperidine odor was detected and this ethanol distillate was adjusted to pH 3 with hydrochloric acid and concentrated again to a small volume in vacuum.

When 30% KOH (in water) was added to a portion of the concentrates, a strong unpleasant odor was detected, consequently the alkaline solution was extracted with ether and this ether extracted solution was dried with anhydrous sodium sulfate, and its concentrate was identified by paper chromatography using *n*-butanol-acetic acid-water (4 : 1 : 2) at room temperature for twenty hours and followed by spraying with ninhydrin (0.2% in ethanol) and exposing to vaporized iodine, both R_F values (0.54) of authentic piperidine and of separated

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1) Y. Obata and T, Yamanishi, *Nogaku* (*Agriculture*), **1**, 537 (1947).

2) Y. Obata and T. Yamanishi, *J. Agr. Chem. Soc. Japan*, **23**, 154 (1949).

3) Y. Obata and T. Yamanishi, *ibid.*, **24**, 477 (1951).

4) Y. Obata and H. Horitsu, *ibid.*, **32**, 615 (1958).

5) Y. Obata and H. Horitsu, This Bulletin, **22**, 153 (1958).

6) Y. Obata and H. Horitsu, *ibid.*, **23**, 186 (1959).

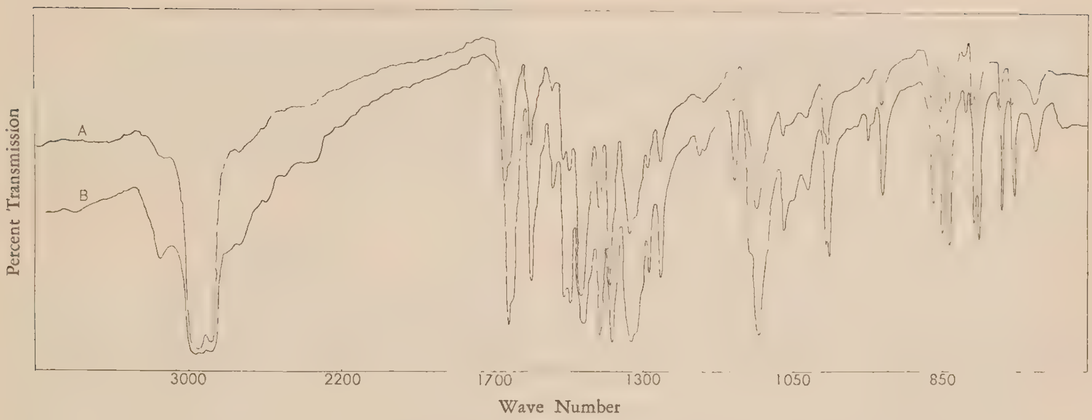
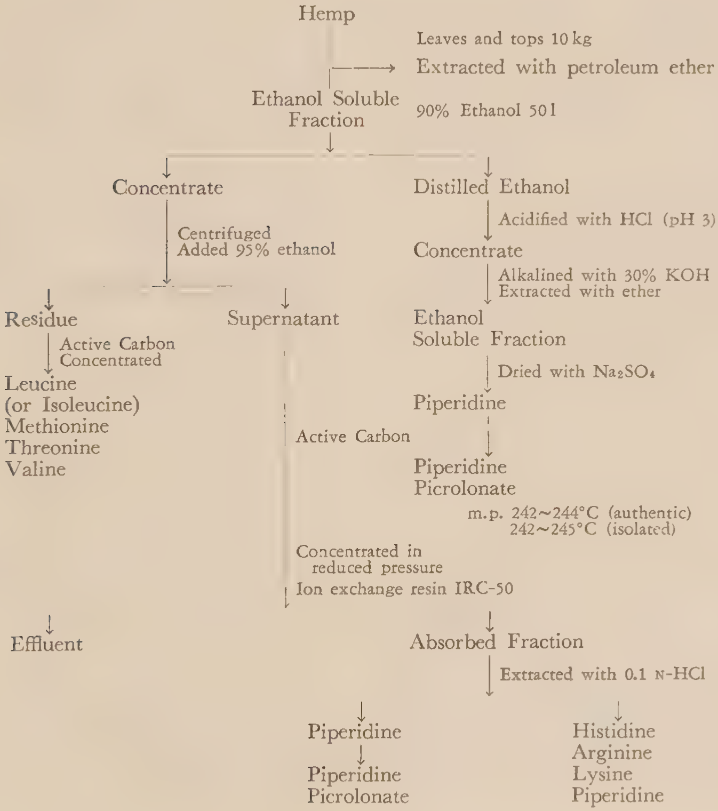


FIG. 1.

A: The infrared absorption spectrum of authentic piperidine picrolonate. (m.p. 242~244°C).
B: The infrared absorption spectrum obtained from hemp plant. (m.p. 242~245°C) (Nujol).

DIAGRAM



piperidine were found to be the same.

Preparation of Piperidine Picrolonate:

Piperidine picrolonate was prepared to identify the component detected by the chromatography as described above.

At first a portion of concentrates, acidified with hydrochloric acid, was placed in 100 ml egg shaped flask and alkalined by adding 30% KOH. This flask was heated in the oil bath over 120°C for about 30 minutes and the evaporated free base was led to a test tube containing alcoholic solution saturated with picrolonic acid. This test tube was allowed to stand at room temperature over night. The red crystals of picrolonates obtained were filtered and dried up in a vacuum desiccator. The melting point of these crystals were measured by a micromelting point apparatus, comparing it with authentic piperidine picrolonate, the former was 242∼245°C, the latter was 242∼244°C. The infrared spectra of these two derivatives in Nujol Mull were found to be coincident as shown Fig. 1 and *Anal.*, found: N, 20.08, calcd. for $C_{15}H_{19}N_5O_5$: N, 20.06%.

Isolation and Identificasion of Combined Piperidine and Several Amino Acids:

When 95% ethanol was added to residue evaporated and cooled in refrigerator for a few days unknown crystals were obtained. These crystals were recrystalized several times from ethanol and filtered and chromatographed. The following amino acids; leucine (or isoleucine), valine, methionine, threonine were detected with ninhydrin (0.2% in ethanol).

The mother liquid was treated with active carbon and then supernatant was passed through ion exchange resin IRC-50 and eluted with 0.1 N-HCl. A portion of the elute was chromatographed after concetration to a small volume. Consequently piperidine was detected by the paper chromatography with spraying ninhydrin as well as arginine, histidine and lysine.

When 30% KOH was added to the elute it gave off a strong piperidine odor, therefore, piperidine was isolated and identified by the same method of making derivatives of piperidine picrolonate as described above.

SUMMARY

In order to isolate and identify the unpleasant odor of wild hemp leaves, tops of the plant in its flowering time was extracted with petroleum ether at first and the residue was extracted with 90% ethanol. In this ethanol soluble fraction piperidine contained in free and combined form in hemp was isolated and identified by means of the paper chromatography analysis and infrared spectra.

The melting point of the picrolonate derived from wild hempen piperidine was 242∼245°C and that of authentic piperidine was 242∼244°C. The infrared spectra of both picrolonates were found to be coincident and *Anal.*, found: N, 20.08, calcd. for $C_{15}H_{19}N_5O_5$: N, 20.06%.

Acknowledgements. The authors wish to express their thanks to Mr. T. Ōnishi, Department of Pharmacology, Hokkaido University for the infrared analysis. They are also greatly indebted to Dr. Y. Matsuoka, Eizai Co., Ltd., for the microanalysis

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 673~681, 1960]

Effect of Gamma-ray upon Food Microorganisms

Part VI. Studies on the Effect of Gamma-ray upon *E. coli* in Components of Varied Meats

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Received June 2, 1960

The author has carried out a series of studies on the effects of various conditions on the survival of *E. coli* irradiated with cobalt-60 gamma-ray. This report deals with the survivals of the strain irradiated in the medium containing each of the components of various types of fish-meats and meats.

INTRODUCTION

In the experiments so far executed, the cells of *E. coil* were irradiated in the artificial media, and the survival-ratios in the media were investigated[1~5]. However, in case of the practical application of irradiation on foodstuffs, the influences of the components of foodstuffs should be more complex than those of the artificial medium. Therefore, the results obtained in the artificial medium must not be believed as same as that in the natural food. Regarding the lethal effect of radioactive rays upon natural flora, Proctor has conducted studies concerning the action of X-ray on ground beef, and observed that the bacterial counts were reduced as a result of irradiation[6], and the author reported that microorganisms in packaged fish-cakes and raw tuna meat have entirely disappeared by gamma-ray irradiation with a high dose[7]. However, the results obtained from natural flora in natural food are so complicated that it is difficult to find the relation between the protective effect of food components and survivals of the strain. The components of food are complicated very much, i.e., they consist of various types of protein, carbohydrate, oil and fat, mineral, vitamin and enzyme, etc. The influences of each or these combinations should be investigated. In this experiment, the studies undertaken are limited to survivals of *E. coli* in the food components, the results of which are presented here.

EXPERIMENTAL

The strain used in this experiment was *E. coli* strain YC 1, one of the stock cultures of the School of Medicine, Keio University. The radiation source used was cobalt-60, of 40 curies, preserved at the School of Medicine, Keio University. The duration of irradiation was 1 hour at room temperature in the range of 18°C to 20°C. The bacterial concentration in the used medium was arranged at the range of 800 to 1,500 cell/ml.

The used food components were various types of meat. In this case, it was impossible to make the cells of the strain in the used samples of meat uniformly. The preparation of the samples was made as follows: The meat was homogenized and suspended in a melted pure agar solution, and then cells of *E. coli* were suspended uniformly. As a result of supplemental experiments, it

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1) W. Watanabe, This Bulletin, **22**, 68 (1958).
2) W. Watanabe, *ibid.*, **22**, 255 (1958).
3) W. Watanabe, *ibid.*, **23**, 73 (1958).
4) W. Watanabe, *ibid.*, **24**, 75 (1960).
5) W. Watanabe, *ibid.*, **24**, 84 (1960).
6) B. E. Proctor and H. Farm, Research Reports on Quarter Master Contract Projects from July 1, 1942 to June 30, 1943, by M.I.T., p. 217.
7) W. Watanabe et al., *Jap. Soc. of Scientific Fisheries*, **20**, 1000 (1955) (in Japanese).

revealed that a mixture of 1.5% of agar and 10% of meat could be used as an irradiating medium.

The used meats in the experiment were as follows:

(a) Non-fatty tuna-fish meat.
(b) Fatty tuna-fish meat.
(c) Tuna-fish blood meat.
(d) Halibut meat.
(e) Salmon meat.
(f) Shark meat.
(g) Shrimp meat.
(h) Cuttle-fish meat.
(i) Oyster meat without ligament.
(j) Small-neck clam without ligament.
(k) Non-fatty beef.
(l) Non-fatty pork.
(m) Non-fatty chicken.

In the course of the experiment, non-contaminated meats should be prepared at first. In case of such a big meat block as tuna-fish, the non-contaminated raw meat can be easily obtained, but in case of other meats, the non-contaminated raw meat cannot be prepared. In case of using heat processed meat, the heat-sterilized meat would be easily prepared. Today, the only method to prepare the sterilized raw meat is by means of gamma-ray irradiation. However, there is some possibility to change small parts of meat components by gamma-ray irradiation and to influence the change upon survivals of the strain. Therefore, the effect of a gamma-ray irradiated meat upon survivals of *E. coli* should be compared with that from a non-contaminated raw meat.

Besides, in case of incubation of the irradiated cells of *E. coli*, it is probable that small amount of meat components which are contained in a nutrient agar medium for incubation helps to recover the cells from irradiation damage. The irradiated samples were diluted ten-fold by saline in the cup of homogenizer, then 2 ml of the mixture was incubated in 15 ml of a nutrient agar medium at 37°C for 48 hours. In case of samples containing 10% of meat, the irradiated bacteria was, then, incubated in a nutrient medium containing 2 ml of the 1% meat suspension. In case of the control, the nutrient medium used for incubation contained no meat. Therefore, the recovering effect of small amount of meat components on the irradiated cells in nutrient medium should be investigated. The procedure and results are described in detail in each section below.

RESULTS AND DISCUSSION

(1) Experiment 1

Eight gram of a raw tuna-fish meat vacuum-packaged with poly-ethylene films was irradiated with cobalt-60 gamma-ray at the distance of 5 cm from the radiation source for 45 hours. After irradiation the appearance of the irradiated raw tuna-fish meat was good due to the vacuum-packaging, but only off flavor was observed in the sample. Besides, 8 g of non-contaminated raw meat was prepared from the same block of tuna-fish meat by means of the ordinary preparation method. Both meats were homogenized in the distilled water in a sterile cup and in this case the suspensions were prepared to contain 20% of each meat. Then the mixture was put into 1.5% pure agar solution. The used concentrations of non-irradiated raw meat were 10, 5, 1, 0.5 and 0.1%. The concentrations of the irradiated raw meat were 10, 5 and 0.5%. The cells of *E. coli* were suspended in this mixture. One ml of the mixture thus prepared was pippeted into each of small Petri-dishes.

All the samples were irradiated for 1 hour at room temperature. The irradiated samples were homogenized and ten-fold diluted in the distilled water. Two ml of the mixture was incubated in 15 ml of a nutrient agar medium at 37°C for 48~72 hours. The results are shown in Fig. 1. The left part of this figure shows the results from a non-irradiated raw meat. As seen in the figure, the higher concentrations of meat components resulted in higher survivals of the strain. The results from an irradiated meat are shown in the right figure, in this case the dotted lines mean the survival-curves from the non-irradiated meat. From the results shown in these figures, the effects of both meats upon surviuals of *E. coli* would be assumed almost the same. Therefore, in the experiments in respect to the effect of raw meats, were sterilized by gamma-ray irradiation prior to preparation of samples.

(2) Experiment 2

The recovering influence of the presence of a small amount of meat in a incubating medium upon survivals of the irradiated *E. coli* was investigated in this section.

The samples containing *E. coli* in only a pure

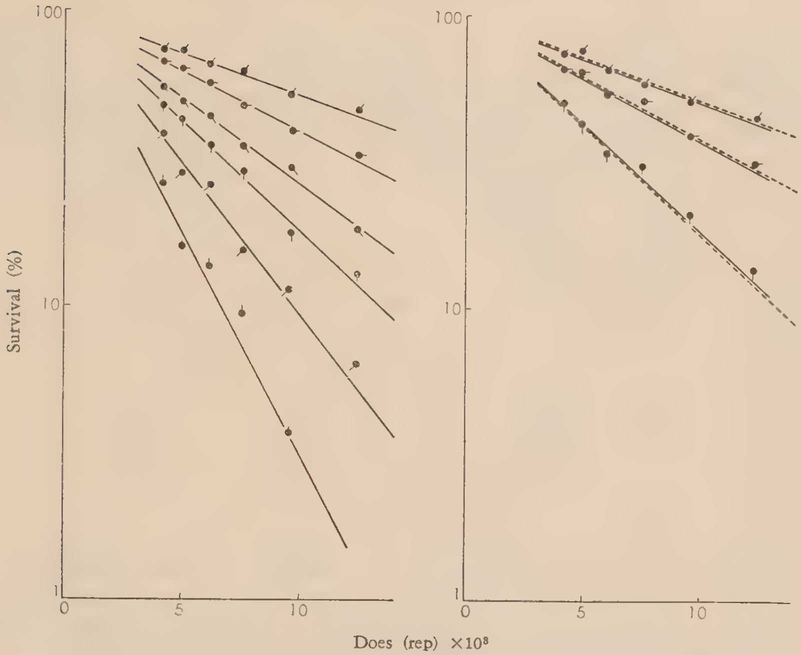

FIG. 1. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Truna Non-fatty Meat.

Left: raw meat, non-irradiated; ● 0% ● 10% ● 5%
Right: raw meat, irradiated ● 1% ● 0.5% ● 0.1%
------ raw, non-irradiated

agar medium were irradiated for 1 hour at room temperature. The irradiated samples were divided into two groups. One group was incubated in only a nutrient agar medium, and the other group was incubated in a nutrient agar medium containing 2% of tuna-fish meat. The used meats were previously a gamma-ray sterilized raw meat and a heat-sterilized meat. The heated meat was prepared by means of boiling a meat-suspension for a few minutes.

The results are diagrammed in Fig. 2. The left part shows the results obtained from the raw meat, and the right those from the heated meat. As it is seen in these figures, the survivals obtained from the samples containing 2% of seems to be slightly higher than the control. But, the differences in both survivals were very small. In case of the 10% sample, the incubation medium contained meat components at the concentration of less than 0.2%, therefore, it was assumed that differences of survivals owing to this recovering effect would be negligible in this experiment.

(3) **Experiment 3**

Each of all the meats was vacuum-packaged and irradiated with gamma-ray. The irradiated meat was homogenized and suspended in distilled water at the concentration of 20%. The suspension was divided into two cups. By using the content in one cup, the agar mixtures containing raw meat at the concentrations of 10, 5 and 0.5% respectively were prepared. The suspension in the other cup was heated at 100°C for 10 minutes and homogenized again. In case of a heated suspension, the used concentrations were also 10, 5 and 0.5% respectively. The used concentrations of *E. coli* were in a range of 800 to 1,500 cell/ml. The samples were diluted and

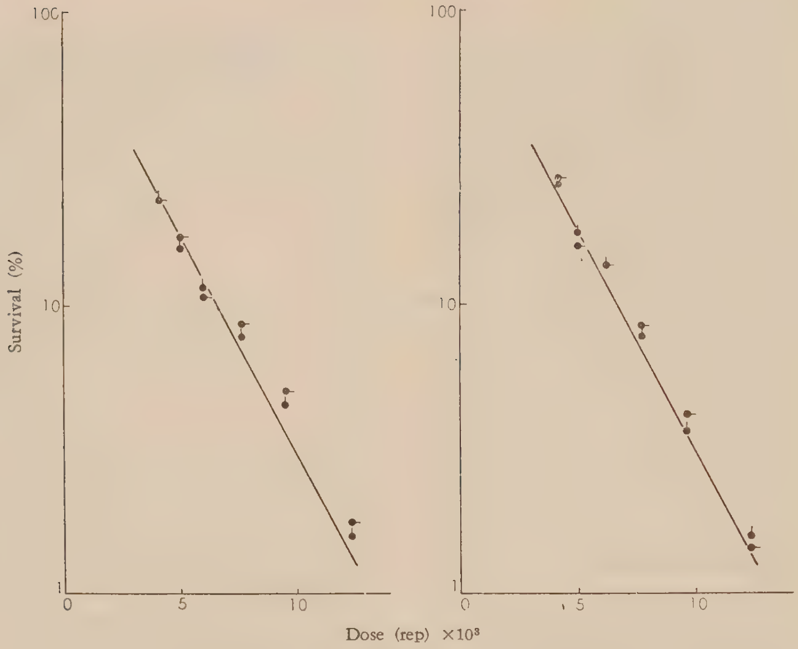

FIG. 2. Survivals of *E. coli* Incubated in a Nutrient Medium Containing Tuna-fish Meat after Gamma-ray Irradiation.
Left: raw, Right: boiled

incubated after 1 hour irradiation at room temperature. The control samples containing no meat were only 1.5% of a pure agar medium. The non-irradiated samples were treated in the same manner except for irradiation procedure.

The survival-ratios and survival-curves obtained from samples containing each of raw and heated meat are diagrammed in the following figures. Those from three kinds of tuna-fish meats are shown in Fig. 3. The left one in this figure shows the results from non-fatty tuna-fish meat, the middle from fatty tuna-fish meat, and the right from tuna-fish blood meat. In Fig. 3, the dotted lines and the black plots show the survivals from the boiled meat. The results from three samples of halibut, salmon and shark are diagrammed in Fig. 4, showing the results with halibut in the left figure, salmon in the middle figure and shark in the right figure respectively. In both the cases of shrimp and cuttle-fish meats, the results are diagrammed in Fig. 5. The left figure shows those from shrimp and the right demonstrates those from cuttlefish. The survival-ratios of both oyster and small-neck clam are diagrammed in Fig. 6. In cases of varied three meats, the results are diagrammed in Fig. 7. It shows the survivals with beef on the left, pork on the middle and chicken on the right.

From these results, in every case the higher concentrations of meat resulted in higher survivals of the strain, and in case of the same meat and concentration, the survivals from the raw meat were higher than those from the heated meat. However, the different meats showed the different protective effects, and it was impossible to compare these 26 kinds of meats with each one another. In the semi-log paper, the tendency of a slope of the survival-curve, being a straight line, shows its gamma-ray resistivity[1,4]. Each of *Sin*-values between the survival-curve and the vertical line obtained from these survival-curves

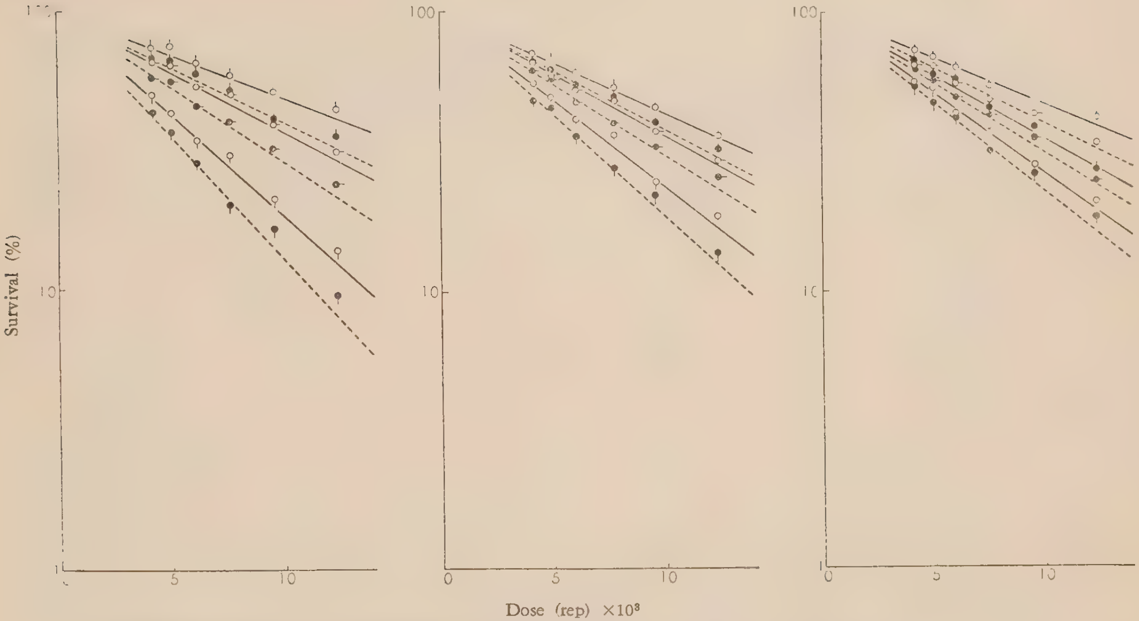

FIG. 3. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Varied Tuna-fish Meats.

Left: non-fatty meat Right: blood meat Middle: fatty meat

ȯ 10% raw o- 5% raw ♀ 0.5% raw ● 10% bioled

•- 5% boiled ♀ 0.5% boiled —— raw ---- boiled

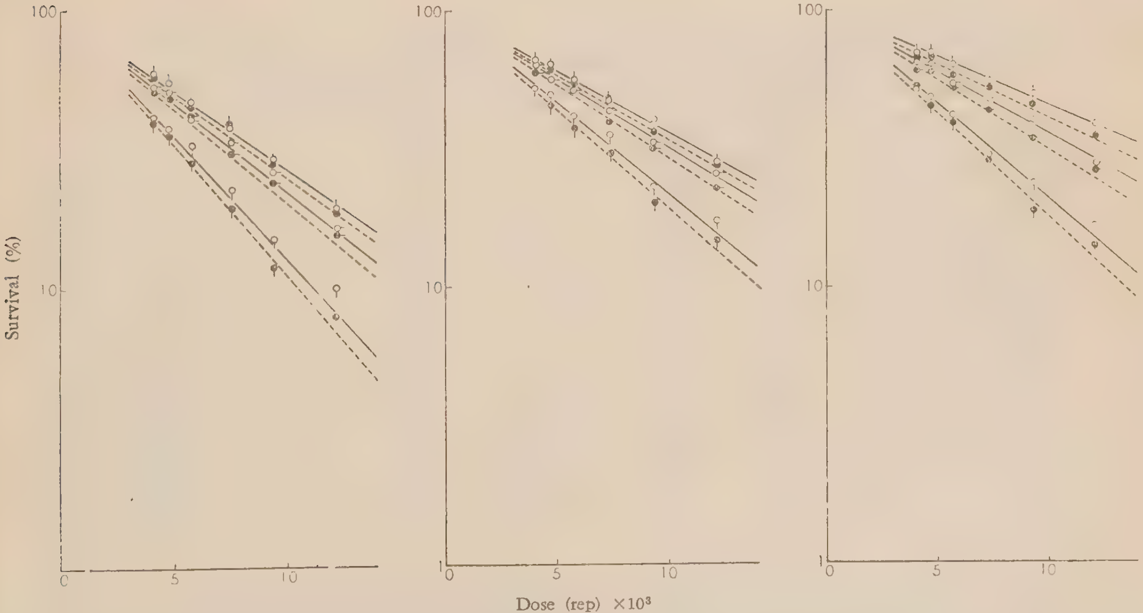

FIG. 4. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Varied Fish Meat.

Left: halibut Right: shark Middle: salmon

ȯ 10% raw o- 5% raw ♀ 0.5% raw ● 10% boiled

•- 5% boiled ♀ 0.5% boiled —— raw ---- boiled

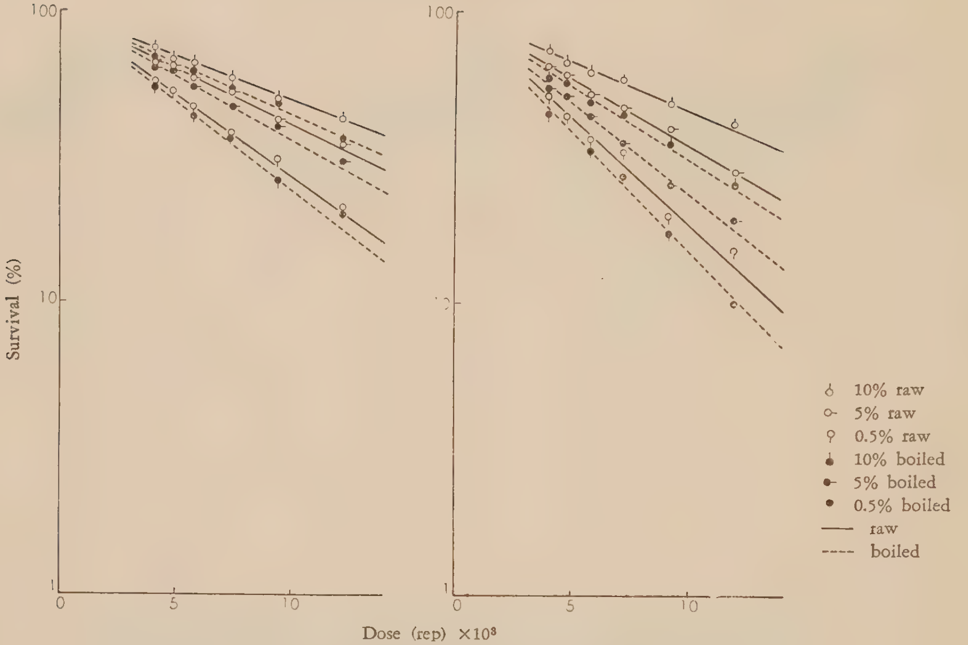

FIG. 5. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Varied Muscles.
Left: shrimp Right: cuttle-fish

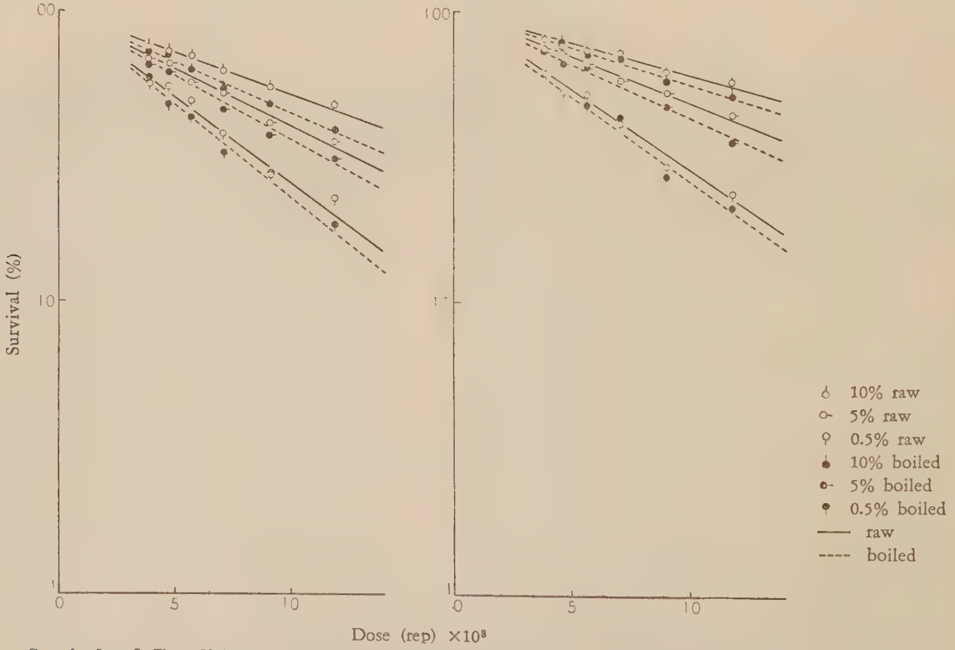

FIG. 6. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Varied Muscles.
Left: oyster Right: small-neck clam

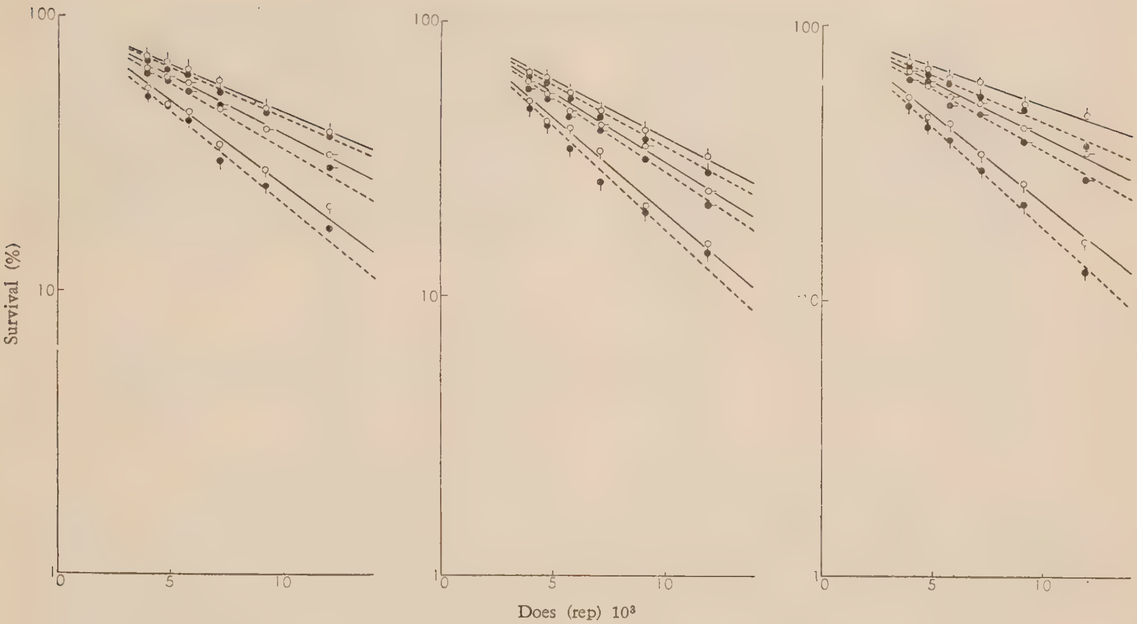

FIG. 7. Survivals of *E. coli* Irradiated with Gamma-ray in a Pure Agar Containing Varied Meats.

Left: beef Middle: pork Right: chicken

ό 10% raw ○– 5% raw ♀ 0.5% raw ● 10% boiled

●– 5% boiled ♀ 0.5% boiled —— raw ---- boiled

TABLE I. *SIN*-VALUE OBTAINED FROM SURVIVAL-CURVES OF *E. COLI* IRRADIATED WITH GAMMA-RAY IN A PURE AGAR MEDIUM CONTAINED DIFFERENT KINDS OF FOOD COMPONENT AT THE BACTERIAL CONCENTRATIONS OF 10³ cell/ml ORDER

Meat	Raw 10%	Raw 5%	Raw 0.5%	Boiled 10%	Boiled 5%	Boiled 0.5%
Tuna, non-fatty	.9336	.8788	.7431	.8988	.8290	.6756
Tuna, fatty	.9100	.8746	.7934	.8870	.8480	.7490
Tuna, bloody	.9239	.8988	.8192	.8746	.8526	.7934
Halibut	.8141	.7826	.6756	.8090	.7660	.6561
Salmon	.8746	.8526	.7716	.8704	.8387	.7660
Shark	.9171	.8746	.7716	.9026	.8526	.7373
Shrimp	.9336	.9026	.8192	.9171	.8829	.8039
Cuttle-fish	.9025	.8746	.7431	.8526	.7986	.7071
Oyster	.9397	.9026	.8141	.9205	.8829	.7934
Small-neck Clam	.9636	.9304	.8387	.9537	.9171	.8141
Beef	.9304	.8988	.8141	.9171	.8829	.7826
Pork	.8949	.8526	.7716	.8870	.8434	.7490
Chiken	.9455	.9026	.7986	.9272	.8788	.7490

was calculated, which are shown in Table I, where the large *Sin*-value means the marked protective effect. From this table, it was observed that, in case of a raw meat, a small-neck clam, an oyster and a shrimp meats belonged to the group with a high protective effect, and a halibut, a salmon and a pork meats belonged to the group having a low protective effect. In

TABLE II. COMPONENTS OF MEATS

Meat	Water %	Crude Fat %	Crude Protein %	Amino-N mg/g
Tuna, non-fatty	75.02	1.02	23.56	0.17
Tuna, fatty	63.94	13.96	21.88	0.13
Tuna, bloody	71.24	2.87	25.63	0.14
Halibut	79.07	0.76	19.69	0.03
Salmon	76.12	3.66	19.63	0.09
Shark	70.18	6.02	24.13	0.23
Shrimp	77.54	1.43	19.75	3.71
Cuttle-fish	76.32	1.65	19.80	1.68
Oyster	80.45	3.49	8.38	3.16
Small-neck Clam	85.91	1.55	10.25	2.10
Beef	75.69	1.81	21.56	0.96
Pork	76.49	2.92	20.38	0.79
Chiken	73.65	1.30	24.50	1.21

case of a heated meat, a small-neck clam, an oyster and a shrimp meats belonged to the group having higher protective effect, and a cuttle-fish, a halibut, non-fatty tuna-fish and a salmon meats belonged to the group with a lower protective effect. As seen from these classifications, a small-neck clam, an oyster and a shrimp meats would be more protective, and a halibut and a salmon meats would be less protective on the survivals of the irradiated *E. coli.*

DISCUSSION

On comparison of the protective effects of meat components, the tendencies of the protective effects of various meats differed from each other. Therefore, the reason of these protective effects should be investigated. The results of the analyses are shown in Table II. As seen in the table, in respect to the water contents, the crude fat contents and the crude protein contents, no correlation could be observed. However, some relation could be recognized in respect to the amino-type nitrogen contents.

A small-neck clam, an oyster and a shrimp meats belonged to the group which has a higher protective effect, and the determined values by the Van Slyke method are 2.10 mg/g, 3.16 mg/g and 3.71 mg/g respectively. Regarding the values obtained from the group with a lower protective effect, the value in case of a halibut meat was 0.03 mg/g and with a salmon meat 0.09 mg/g. From these facts, it can be at least observed that there is some correlation between the protective effect of meat upon survivals of *E. coil* and the substances analysed as the amino-type nitrogen by means of the Van Slyke method.

SUMMARY

(1) The survival-ratios of *E. coli* irradiated in each of the various types of meats and fish meats differed from each one another.
(2) Small-neck clam, oyster and shrimp meats belonged to the group having high protective effect on the survivals of the strain.
(3) Halibut and salmon meats belonged to the group having low protective effect.
(4) In case of the same meat and the same concentration, the survivals in a raw meat were higher than those in a heated meat.
(5) There could be observed some correlation between this protective effect and the substances analysed as the amino-type nitrogen by means of the Van Slyke method.

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Effect of Gamma-ray upon Food Microorganisms

Part VII. Studies on the Effect of Gamma-ray upon Food Bacteria under Some Conditions

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Received June 2, 1960

The author has carried out a series of studies on the effects of gamma-ray on a single strain of *E. coli* irradiated with cobalt-60 in the previous reports. This report deals with the effects of three conditions which showed the higher survivals of *E. coli* upon other food bacterias. In this experiment, a pure agar medium has been used for irradiation besides a nutrient agar medium.

INTRODUCTION

In the previous reports, a single strain of *E. coli* was used as a strain of bacteria, and studies were performed concerning the effect of gamma-ray upon survival-ratios of this strain[1~6]. However, it is well-known that in respect to the strain of *E. coli* some variants on mutants of the strain show different gamma-ray resistivities or different survival-ratios under the same condition, as compared with those of other strains[7~9]. It is a matter of course that various types of food microorganisms show different gamma-ray resistivities. Therefore, in this experiment, the test was made in regard to the survival-ratios obtained from irradiated samples containing other types of bacteria under two or three conditions showing higher survivals of *E. coli*, the results of which are presented below.

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1) W. Watanabe, This Bulletin, **22**, 68 (1958).
2) W. Watanabe, *ibid.*, **22**, 255 (1958).
3) W. Watanabe, *ibid.*, **23**, 73 (1958).
4) W. Watanabe, *ibid.*, **24**, 75 (1960).
5) W. Watanabe, *ibid.*, **24**, 84 (1960).
6) W. Watanabe, *ibid.*, **24**, 673 (1960).
7) S. Sasaki, T. Akimoto and W. Watanabe, *Jap. Radioisotopes*, **3**, 2, 40, (1954).
8) H. Kadota, H. Miyoshi and H. Shibata, *Jap. Soc. Sci. Fisheries*, **24**, 12, 1001 (1959).
9) H. Kadota, H. Miyoshi and H. Shibata, *Bulletin of the Research Institute for Food Science*, Kyoto University, **22**, July (1959).

EXPERIMENTAL AND DISCUSSION

The strains used were *E. coli*, one of the stock cultures preserved at the School of Medicine, Keio University, used in the previous reports, and three strains of bacteria were used, i.e., *A. aerogenes*, isolated from fish-cake by the author, *Prot. vulgaris* and *Pseud. aeruginosa* stocked at University of Tokyo. *A. aerogenes* was chosen for the reason of its high gamma-ray resistance, *Port. vulgaris* was chosen for the reason of the medium resistance, and also *Pseud. aeruginosa* was used because of its lower resistance.

The conditions showing higher survivals of irradiated *E. coli* in the previous reports were the addition of each of organic acids, thio-urea, amino-acids and proteinous materials in the medium used for both irradiation and incubation. In this experiment, succinic acid was chosen among organic acids, and three amino-acids, arginine, threonine and proline were used. Arginine was chosen for the reason of its non-effect in a synthetic medium, threonine for its medium effect, and proline for its marked protective effect on the survivals of *E. coli*. Besides, thio-urea, which is well-known as a protector against radio-active ray, was also used.

The used media were both a nutrient agar medium and a pure agar. In case of amino-acids only a pure agar was used. Of course, in case of using a pure agar, non-nutrient, the irradiated medium was covered with a nutrient agar medium prior to incubation.

The preparation method was same as in the previous reports. All the samples were irradiated at room temperature in a range of 19°C to 20°C for 2 hours. After irradiation all the irradiated samples and the non-irradiated control samples were incubated at 37°C for 48 hours. The procedures and results are described in detail in each section below.

1) Experiment 1

In respect to the influence of the addition of succinic acid, there are some reports concerning the irradiation in a succinic acid solution experimented by Stapleton. He has reported that the survivals of *E. coli* irradiated in succinic acid solution were higher than the control, and in this case the protective effect of this substance on survivals of the strain decreased owing to the addition of Prussic acid, Malonic acid and Iod-acetic acid into the medium[10]. The author has also reported that the presence of succinic acid in the used nutrient medium resulted in protective effect on the survivals of the irradiated *E. coli*[3].

In case of the addition of succinic acid in the used medium, when the concentration of this chemical substance was higher than a certain limit, no colony developed in the medium after incubation at 37°C. And, this limit of concentration of succinic acid with some strain of bacteria differed from each other. In case of using a nutrient agar medium, the limit with *E. coli* was 0.5%, and the limit of *A. aerogenes*, *Prot. vulgaris* and *Pseud. aeruginosa* turned to be 0.1%. Then, the used concentrations were the ten-fold dilutions of their limits, i.e., 0.05% with *E. coli* and 0.01% with other three strains. All the survival-curves are diagrammed in Fig. 1. In this figure, the dotted lines show each survival-curve obtained from samples containing no succinic acid. From this figure, the influence of the addition of succinic acid in the nutrient medium turned to be more or less protective upon survivals of the other type of bacteria as well as those of *E. coli*.

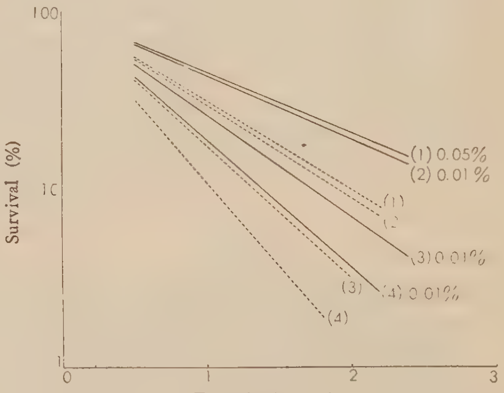

FIG. 1. Survival-curves of Bacteria Irradiated in Nutrient Media in which Contained Succisnic Acid.

(1) *E. coli* (2) *A. aero.*
(3) *Pr. vul.* (4) *Ps. aerug.*

In case of using a pure agar medium, the different limits of the concentration of succinic acid were also found. The used concentrations added to a pure agar were 0.01% with *E. coli*, 0.05% in case of *A. aerogenes*, 0.005% with *Prot. vulgaris*, and 0.05% in case of *Pseud. aeruginosa*. All the results are diagrammed in Fig. 2. As shown in this figure, a little higher survivals of these strains were observed as in case of using a nutrient medium, showing highest survivals with *E. coli*.

(2) Experiment 2

In respect to the protective effect of the presence of thio-urea, the author has reported the remarkable effect on the survivals of *E. coli* in a nutrient medium[1].

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[10] G. E. Stapleton, *Bact. Reviews*, **19**, No. 1, 26 (1955).

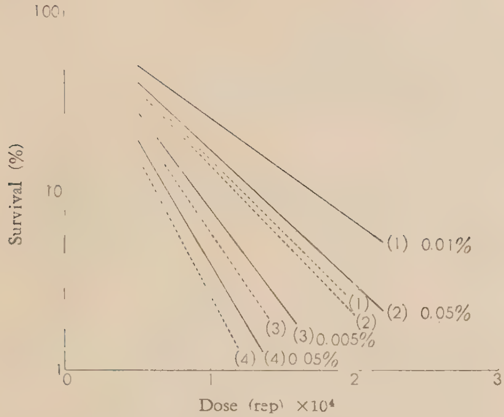

FIG. 2. Survival-curves of Bacteria Irradiated in Pure Agar Media in which Contained Succinic Acid.

(1) *E. coli* (2) *A. aero.*
(3) *Ps. vul.* (4) *Ps. aerug.*

It is well-known that thio-urea is a bacteriostatic or bacteriocidal agent and that bacteria cannot grow in the medium containing high concentrated thio-urea. Therefore, the limit concentration with each strain was tested previously. The used concentrations of thio-urea in a nutrient medium were 0.1, 0.3 and 0.5% in case of *E. coli*, 0.05% in case of *A. aerogenes*, *Prot. vulgaris* and *Pseud. aeruginosa*. All the survival-curves are presented in Fig. 3, showing higher survivals of all the strains of bacteria when thio-urea was added. In case of *E. coli*, the higher concentration resulted in higher survivals of the strain. From these results, it was recognized that the addition of thio-urea in medium within a certain limit of concentration turned to be protective upon survivals of three strains of food bacteria as well as *E. coli*.

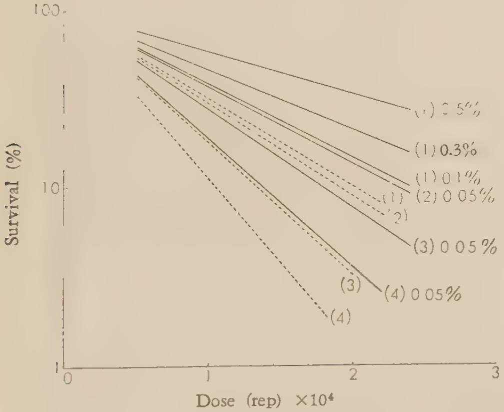

FIG. 3. Survival-curves of Bacteria Irradiated in Nutrient Media in which Contained Thio-urea.

(1) *E. coli* (2) *A. aero.*
(3) *Pr. vul.* (4) *Ps. aerug.*

Next, in the same manner, the experiments using a pure agar medium were undertaken. The used concentrations added to a pure agar were 0.1 and 0.05% in case of *E. coli*, 0.05% in both cases of *A. aerogenes* and *Pseud. aeruginosa*, and 0.01% in case of *Prot. vulgaris*. The results are presented in Fig. 4. In case of *E. coli*, the survivals obtained from samples containing 0.05% resulted in rather lower than those of the control, despite showing higher survivals in those containing 0.1%. In cases of other three strains, all the survival-curves turned to be lower than the dotted lines from control. The reasons for these results are not known, but two reasons, i.e., the bacteriocidal effect and the effect of S-compound of thio-urea, can be assumed. In cases of other strains, as they could not grow in higher concentration of thio-urea, this phenomenon could not be observed.

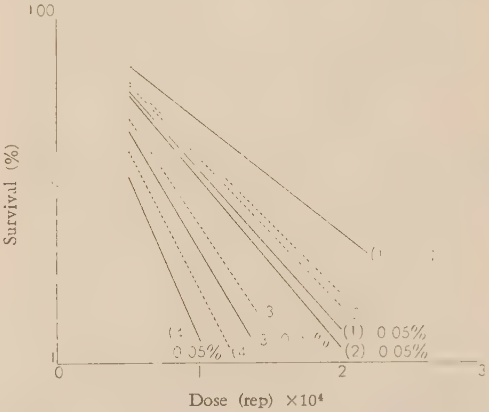

FIG. 4. Survival-curves of Bacteria Irradiated in Pure Agar Media in which Contained Thio-urea.

(1) *E. coli* (2) *A. aero.*
(3) *Pr. vul.* (4) *Ps. aerug.*

Consequently, it is assumed that the higher survivals in a nutrient medium owed to the effect of S-compound and the lower survivals in a pure agar owing to the bacteriocidal effect of thio-urea. In respect to the results in case of a pure agar, one reason would be considered that owing to the used concentration of ten-fold dilution of the highest limit, the addition of this small amount of the agent resulted in a little effect on the physiological character of the irradiated strain which shows normal colonies grown in case of non-irradiation. Another reason would be assumed that the addition of this substance disturbed the normal growing of the irradiated strain which shows normal growth before irradiation.

(3) Experiment 3

In respect to amino-acids in the previous experiments, a synthetic agar medium for *E. coli* was used in order to eliminate the influence of initial proteinous materials. However, this synthetic medium could not be used in cases of three strains of bacteria. Therefore, in this experiment a pure agar (non-nutrient) was used. The amino-acids included arginine, threonine and proline, and the used concentration in the medium was equally 0.05%. The irradiated and non-irradiated media were covered with a nutrient agar medium prior to incubation.

The results are illustrated in Fig. 5. As seen from the results, all the survival-curves of the strains turned to be almost the same as those from the control. From these results, no influence of the addition of amino-acid in the pure agar medium can be observed on the survivals of these four strains of food bacteria.

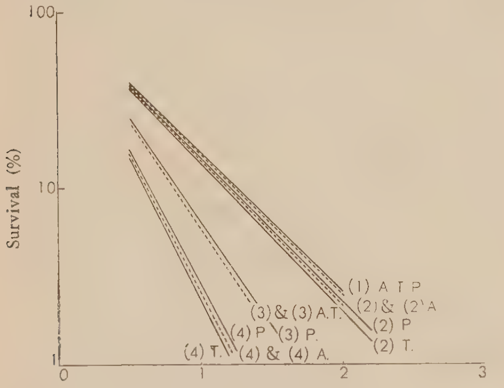

FIG. 5. Survival-curves of Bacteria Irradiated in Pure Agar Media in which Contained Different Amino-acids.

A: arginine, T: threonine P: proline

(1) *E. coli* (2) *A. aero.*
(3) *Pr. vul.* (4) *Ps. aerug.*

SUMMARY

In case of using a nutrient agar medium, all three conditions resulted in protective upon three strains same as in case of *E. coli*. However, in case of using a pure agar, the conditions which show higher survivals of *E. coli* in a nutrient medium were divided into three conditions. The first showed the protective effect same as in case of nutrient medium. The second showed no influence, and the last showed, on the contrary, lower survivals of the strain than those from the control.

The results of this experiment are summarized as follows:

(1) Three conditions which were the addition of each of succinic acid, thio-urea and amino-acid in a nutrient agar medium showed the same protective effect on the survivals of the above three strains as in case of *E. coli*.
(2) In case of using a pure agar medium for irradiation, the effects of these conditions differed from each other. Higher survivals, similar survivals and lower survivals of the strains were observed.
(3) The addition of succinic acid in a pure agar resulted in higher survivals of the straines.
(4) The addition of thio-urea in a pure agar caused lower survivals.
(5) The addition of amino-acid in a pure agar brought no influence on the survivals.
(6) In case of *E. coli* in a pure agar containing thio-urea, the higher concentration resulted in higher survivals than the control, however, the lower concentration than a certain limit gave lower survivals than those from the control.

Acknowledgement The author wishes to express his cordial thanks to Prof. T. Asai, Department of Agricultural Chemistry, University of Tokyo, and Prof. T. Obara, Department of Agricultural Chemistry, Tokyo University of Education, for their invaluable advices and constant guidances. He is also indebted to Prof. D. Ushiba, Asist. Prof. S. Sasaki, Department of Bacteriology, Keio University for their kind suggestions, and to Prof. H. Kadota, the Research Institute for Food Science, Kyoto University, for his helpful and valuable advice. Thanks are also due to Dr. H. Yamashita, Radium Center of Cancer Institute, for his sound advice concerning radio-isotopes.

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The Condensation of Alkylhalide with Senecioate

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The condensation of alkylhalide with senecioic ester provides a mixture of α-alkyl-α-iso propylidene and α-alkyl-α-iso propenyl acetic acid.

In the previous publications[1,2] the condensation of aldehydes with senesioic ester in the presence of alkali amide was described, where the aldehydes attacks ω-position of the ester and ω-alkylidene senesioic acid are obtained (Fig. 1 [II]).

It is the objective of the present work to study whether the condensation of alkylhalide and senecioic ester under analogous condition might be expected to occur and to decide the structure of the product. From the results of the present work, the following general conclusions may be drawn:

(1) When the carbonium anion of senecioate is present in liquid ammonia, the halide condenses with the ester and alkylated acid is obtained in good yield, without providing alkene or amine derivative which may occur from interaction of halide and alkali amide or ammonia[3].

(2) The alkyl halide attacks at α-position of senecioate and the product is a mixture of α-alkyl α-isopropylidene and α-alkyl α-isopropenyl acetic acid [III].

In the analogy of the aldehyde condensation, it is our first expection that the alkyl halide will attack at ω-position of senecioate and provide ω-alkyl senecioic acid. However, in fact, the prodoct is proved to be α-alkylated acids and their structures are decided as follows:

For example, in the case of the condensation of iso amylbromide with ethyl (Ia) or benzyl (Ib) senecioate, the produced acid absorbes one mole of hydrogene in the presence of paradised charcoal and this saturated acid is identified with 2-isopropyl-5-methyl hexanoic acid (IVa) by comparion of the melting point of the acid amide with the authentic sample.

The infra-red spectrum of the unsaturated acid shows maxima at 1710, 1695 (saturated and unsaturated acid carbonyl stretching), 1645, (carbon-carbon unconjugated double bond streching) 3100, and 855 cm^{-1} (C–H out of plane deformation of $-CH=CH_2$). These data can be correlated consistently with the mixture of α,β- and β-γ-(vinyl type) unsaturated acids. From ozonolysis of the mixture we could obtain acetone as 2,4-dinitrophenyl hydrazone, but formaldehyde was not surely confirmed in the ozonized product.

Using two equivalents of potassium amide[3], a half mole of unreacted senecioic ester is recovered and the residue is divided into two fractions; one is easily hydrolysed and identical with the mixture [III], but the other ester shows strong resistance to alkaline hydrolysis. The infra-red spectra of this ester and its hydrogenated product (absorbed one mole of hydrogen) indicate that this ester may be α,α-dialkylated α-isopropenyl acetic acid.

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[1] M. Matsui et al., *Proceedings of the Japan Academy*, **34**, 220 (1958).

[2] M. Matsui et al., *The Journal of Vitaminology*, **4**, 190 (1958).

[3] C. R. Hauser and T. M. Harris, *J. Am. Chem. Soc.*, **80**, 6360 (1958).

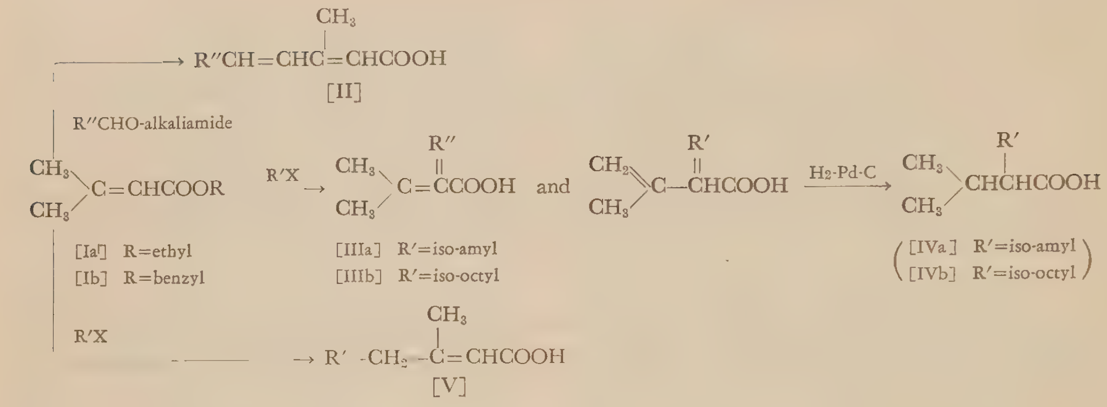

FIG. 1.

EXPERIMENTAL

General Procedure:

In a three necked flask equipped with an efficient stirrer, an out let tube, and a dropping funnel was placed 200 ml of anhydrous liquid ammonia and a catalitic amount of ferric nitrate. To the stirred liquid was added 0.1 gram atom of alkali metal and resultant blue colour disappeared to a colorless solution in 30 minutes. To this solution 0.1 mol of senecioic ester was added in one portion. After stirring 0.5 hours, 0.1 mol of alkyl halide was added dropwise during about 30 minutes and stirring was continued for 3 hours.

Standing overnight at room temperature, ammonia was evaporated and the residue was dissolved in 100 ml of alcohol. After separation of precipitated inorganic salt by filtration the solution was refluxed with potassium hydroxide for six hours. The solvent was evaporated to dryness and the residue water dissolved in water. Acidified with hydrochloric acid, the separated oil was extracted with ether and distilled under reduced pressure.

Condensation of iso-Amylbromide:

1) From 14 g of amylbromide and 13 g of ethylsenecioate 11.5 g (68%) of acid is obtained. b.p. 110~115°/4 mmHg. n_{α}^{20} 1.4500
   Anal. Calc. for $C_{10}H_{18}O_2$: C, 70.54; H, 10.66. Found: C, 70.63; H, 10.84.

   The acid (3.4 g, 1/50 mole) absorbed 480 ml of hydrogen under atomospheric pressure and room temperature in the presence of 0.2 g of paradium charcoal. Saturated acid boils at 135~136°C/16 mm Hg (3.3 g)

   Amide. m.p. 112°C. Mixed melting point with 2-isopropyl-5-methylhexanoic amide shows no depression. Calc. for $C_{10}H_{21}OH$: C, 70.12; H, 12.36; N, 8.18. Found: C, 71.19; H, 11.64; N, 8.84
2) In stead of potassium amide, sodium amide is applied to the reaction. Yield 8.5 g (50%) n_{α}^{20} 1.4500
3) Amylbromide (14 g) is condensed with 17.4 g, of benzyl senecioate. Yield of the acid 10.4 g, which is identified as dihydro-acid to authentic sample.

Condensation of iso Octylbromide:

The yield of α-iso propyl γ-ethyl octanoic acid is 4 g (20%) b.p. 150~152°/12 mmHg. n_{α}^{20} 1.4580
Anal. Calc. for $C_{13}H_{24}O_2$: C, 73.53; H, 11.39. Found: C, 73.09, H, 11.37.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 687~692, 1960]

Studies on Mannan in Wood Pulp

Part IV. Isolation and Purification of Glucomannan

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Received June 6, 1960

Glucomannan was isolated from holocellulose pulp of pine (*Pinus densiflora* Sieb. et Zucc.) using dimethylsulfoxide and aqueous alkali. It was found that there were present also, in the pulp, separate two glucomannans as the possible contaminants. Purification of the glucomannan by extraction with dimethylsulfoxide, the following acetylation and fractionation resulted essentially homogeneous glucomannan triacetate $[\alpha]_D$ $-30.7°$ contained mannose and glucose only in the ratio 36 : 10. Measurements of intrinsic viscosity in the chloroform solution gave $[\eta]$ 0.776 and 0.291 for high and low molecular parts of the acetate respectively. The glucomannan derived its acetate showed $[\alpha]_D$ $-36.5°$ and 78.5% mannose content.

Recently it has been found that coniferous woods and their pulp contained glucomannans which have been studied by Aspinall[1], Dutton[2], Hamilton[3,8], Lindberg[4], Timell[5], Wise[6], Jones[7,10] and their co-workers. In spite of their important contributions in this field, the structural feature of glucomannan remains unclear in many points. Jones[7] reported the presence of galactose residues in the glucomannan but he also considered it as originated from galactomannan. On the other hand, Hamilton[8] thought that the galactose residue is attributed to a separate galactoglucomannan. Further, there are divergences of opinions on the ratio of mannose to glucose, the presence of cellobiose fragment as a constituent of the glucomannan or the configuration and size of the molecule. It may be considered that these questions are all owing to the difficulties of removing the contaminants from glucomannan molecule. In the previous paper[9], the presence of glucomannan in dissolving pulp prepared from pine was recognized by isolation of octaacetyl 4-O-β-D-glucopyranosyl-α-D-mannose from the acetolysis product. This paper is concerned with separation of the glucomannan from the contaminants and characterization of the pure specimen. This also includes the detection of the contaminating hexosans. Polysaccharides constructing hemicellulose were isolated by successive extractions with dimethylsulfoxide and aqueous alkali from holocellulose pulp of pine which was free from pectin and water-soluble polysaccharides. First extraction was effected with dimethylsulfoxide-acetic acid mixture which gave a polysaccharide mixture (I), $[\alpha]_D$ $-39°$, composed of 4-O-methylglucurono-araboxylan[11] and a new type of glucomannan (II). Dimethylsulfoxide-boric acid was then used as extractant and the extracted material was purified by adsorption to Amberite

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1) G. O. Aspinall, R. A. Laidlaw and R. B. Rashbrook, *J. Chem. Soc.*, **1957**, 4444.
2) G. G. S. Dutton and K. Hunt, *J. Am. Chem. Soc.*, **80**, 5697 (1958).
3) J. K. Hamilton and H. W. Kircher, *J. Am. Chem. Soc.*, **80**, 4703 (1958).
4) B. Lindberg and H. Meier, *Svensk Papperstidn.*, **60**, 785 (1957).
5) T. E. Timell and A. Tyminski, *TAPPI*, **40**, 519 (1957).
6) E. Merler and L. E. Wise, *TAPPI*, **41**, 80 (1958).
7) J. K. N. Jones and T. J. Painter, *J. Chem. Soc.*, **1959**, 573.
8) J. K. Hamilton and E. V. Partlow, *J. Am. Chem. Soc.*, **80**, 4880 (1958).
9) T. Koshijima and I. Tachi, This Bulletin, **22**, 11 (1958).
10) J. K. N. Jones and T. J. Painter, *J. Chem. Soc.*, **1957**, 669.
11) J. K. Hamilton, E. V. Partlow and N. S. Thompson, *TAPPI*, **41**, 803 (1958); *ibid.*, **41**, 811 (1958).

IR-A 400. The material eluted from the resin with 1 N ammonium carbonate solution was a new type of glucomannan (II), $[\alpha]_D^{22}-8°$ (water) contained 3.8 parts of mannose and one part of glucose. The constitution of the glucomannan is under experiments. A large part of 4-O-methylglucurono-araboxylan (III), $[\alpha]_D-42°$ was obtained from the residual pulp by the extraction with 1% sodium hydroxide and the usual glucomannan eluted for the first time by the following 10% sodium hydroxide extractions. It was purified using dimethylsulfoxide which dissolved only 4-O-methylglucurono-araboxylan (B), $[\alpha]_D-49°$ and left glucomannan (IV), $[\alpha]_D$ -39°. Mannose and glucose were present, respectively, in a ratio 3.6 : 1 with trace of xylose and galactose in (IV). The glucomannan (IV) precipitated with methanol from its water solution was made free from water by washing with methanol and the methanol-wet material was acetylated twice with excess of acetic anhydride and pyridine. The fully acetylated (IV) was then fractionally precipitated from the chloroform solution with petroleum ether and the pure glucomannan triacetate fractions were combined, $[\alpha]_D-30.7°$ (chloroform) (see Table I). Deacetylation of the acetate with sodium methoxide resulted glucomannan $[\alpha]_D-36.5°$ (10% NaOH) contained 78.5% mannose residue. Paper chromatography showed the presence of mannose and glucose only. The intrinsic viscosity of a solution of fraction (22) and (31) in chloroform was 0.776 and 0.291 100 ml/g respectively. These correspond to average D.P. of 146 and 55 when Staudinger and Daumiller's constant for cellulose triacetate, $Km \cdot 10^4=5.3$ is applied to the glucomannan triacetate. A part of the acetylated glucomannan was fractionated by Jones and Painter's procedure⁷⁾ and divided into fraction (GM) and (X), the latter being fractionally precipitated in the same way as above described (Table II). Fraction (GM) was the glucomannan triacetate $[\alpha]_D-30.6°$ containing a small amount of galactose which, however, had also a tendency to distribute over fraction (X). This result indicates that galactose residues may be originated from the other polysaccharides.

A part of the extract contained (IV) precipitated upon neutralizatson with acetic acid. The precipitate purified with alkali was a separate glucomannan (V) indicating a specific optical rotation of $[\alpha]_D-32°$ and consisted of four parts of glucose and one part of mannose. The results of the fractionation (Table I) indicates that the glucomannan acetate prepared is composed of two or three polysaccharides. It is probable that the acetate of this type of glucomannan $[\alpha]_D-19°$ 12) contaminates the higher molecular part of the present glucomannan acetate.

EXPERIMENTAL

Paper Chromatography

Aniline hydrogen phthalate was used as spray reagent throughout the experiment. For the separation of neutral sugars solvent (a), ethylacetate-pyridine-water 10 : 4 : 3, and for uronic acid fragments solvent (b), ethylacetate-acetic acid-formic acid-water 18 : 3 : 1 : 4 were used respectively. Measurement of the sugar-ratio in the fractionated glucomannan acetate was made on the revealed spots which were cut off from the paper, and weighed after conditioning in desiccator for twenty-four hours. In each of the cases, five series of the chromatogram of the hydrolyzate were developed two times with solvent (a) under the same conditions and the ratios of spot-weights of mannose to glucose were calculated from five specimens. From the average value of the ratios expressed in logarithm, the ratio of the amount of mannose to glucose in the hydrolyzate of the deacetylated glucomannan was determined immediately using the calibration curve I in Fig. 1 which showed that the ratios of spot-weights were proportioned to the ratios of sugar-amounts adsorbed to the paper in a fairly range when both of the ratios were calculated on the corresponding values of glucose and expressed with logarithms. In this way, the ratios of xylose to glucose and galactose to glucose were determined by the curve II and III, respectively.

Preparation of Holocellulose Pulp

Delignification of acetone-extracted wood meal (360 g) was effected by four times chloriting¹³⁾ followed by one treatment with chlorine and monoethanolamine¹⁴⁾. The holocellulese (240 g) contained 4.26% Klason-lignin was

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12) T. Koshijima, *J. Japan Wood Res. Soc.*, in press.
13) T. Koshijima, *ibid* in press.
14) T. E. Timell and E. C. Jahn, *Svensk Papperstidn.*, **54**, 831 (1951).

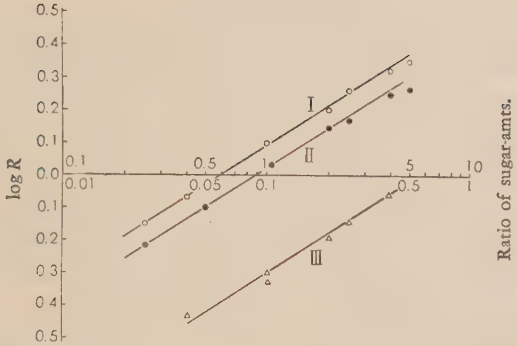

FIG. 1.

For xylose and galactose, abscissa is graduated in one-tenth of the scale designed for mannose.
I. Mannose : glucose.
II. Xylose : glucose
III. Galactose : glucose
R : Ratio of spot-wt.

extracted with 0.5% ammonium oxalate (pH, 6.3) for twenty hours and the water suspension was then beaten in a laboratory beater. This treatment resulted very swollened holocellulose pulp which was soaked in water (10 l) over-night and filtered, water-soluble polysaccharide (A) (3.5 g) being obtained from the filtrate as usual manner. Fraction (A) showed $[\alpha]_D^{20}$ $-37°$ (10% NaOH, c, 1.0) and contained mannose (26.4%), glucose, xylose, arabinose, galactose and 4-O-methylglucuronic acid. The chloriting of the residual lignin in the pulp was repeated more three times using the same reagent and one-half of the reaction time. The resultant pulp (197 g) was washed with water, methanol, benzene and air-dried: Klason-lignin, 1.88; mannose residue, 13.8; Ac, 1.46%.

Isolation of Polysaccharides Constructing Hemicellulose

Air-dried pulp (157 g) was extracted with dimethylsulfoxide-acetic acid and then dimethylsulfoxide-boric acid as described in the previous report[13]. Dimethylsulfoxide-acetic acid extraction yielded a polysaccharide mixture (I) (2.16 g); $[\alpha]_D^{20}$, $-39°$ (1% NaOH, c, 1.0); Ac, 2.41; mannose residues, 22.4%. Paper chromatography (solvent a and b) showed the presence of xylose, arabinose, mannose, glucose, galactose and 4-O-methylglucuronic acid in the hydrolyzate. Dimethylsulfoxide-boric acid extract was composed of a glucomannan (1.28 g) as main product which contained 4.43% Ac. and mannose, glucose, galactose and xylose as component sugars (solvent a). It was treated with Amberite IR-120 and IR A-400 (carbonate form) resin with stirring. The anion-exchanger was washed with water until negative for anthrone and then soaked in 1 N ammonium carbonate solution for one month. The resin was separated from the solution which was evaporated to a small volume in vacuo and precipitated with 10 volumes of ethanol. The resultant white powder (II) was easily soluble in water and was composed of mannose and glucose in a ratio 3.8 : 1 with trace amounts of xylose, yield 1.21 g, $[\alpha]_D^{22}$ $-8°$ (water, c, 1.0), $-4°$ (1% NaOH, c, 1.0). Uronic acid fragments in the hydrolyzate showed the presence of methyl-glucuronic acid and an unknown spot neighboring start line (solvent b).

Dimethylsulfoxide-extracted pulp (240 g) was mixed with 1% sodium hydroxide solution (3 l) and allowed to stand for thirty minutes. The filtrate and washings acidified with acetic acid were combined and the precipitate produced after three days was separated. The clear, light yellow extracts were made precipitate by addition of 3 volumes of methanol, removed by centrifugation, resuspended in methanol and dried, yield 14 g. The bulky white powder (III) showed an optical rotation of $[\alpha]_D^{15}$$-42°$ (10% NaOH, c, 0.9) and the paper chromatography followed by hydrolysis of (III) indicated the presence of a large amount of xylose, arabinose and 4-O-methylglucuronic acid fragments (solvent a and b).

Ten percent sodium hydroxide solution (2.5 l) was added to the residual pulp without drying and nitrogen gas bubbled through the suspension for one hour. Extraction was continued for twenty-four hours and then the extract was filtered and washed with this solution through cloth into the vessel containing acetic acid with external cooling. The acidic extract was turbid and, after five days, the precipitate (Va) produced removed by centrifugation. The residual pulp was extracted again with 10% sodium hydroxide and the centrifugated liquor of two extracts were combined and dialyzed through gel cellophane for two weeks. The water solution was evaporated to one-fifth and precipitated with methanol, the precipitate being removed by filtration, resuspended in methanol, filtered and washed with methanol, yield 34 g (IVa). Paper chromatography of the hydrolyzate showed the presence of a large amount of mannose, glucose, galactose and xylose (solvent a).

The precipitate (Va) was washed with water by centrifugation, resuspended in methanol-water (1 : 3) and the supernatant liquor discarded. This treatment was repeated six times and the final precipitate recovered from a methanol suspension. The methanol-wet precipitate was dissolved in 1% sodium hydroxide, neutralized with acetic acid and the resultant precipitate was centrifuged. It was dissolved again in 10% sodium hydroxide and made precipitate as above. This was repeated once more and the final precipitate was washed with water, 50% methanol and methanol. The product (V) (1.3 g) contained 19.8% mannose residue, indicated $[\alpha]_D^{18}$ $-32°$ (10% NaOH, c, 0.3). On hydrolysis and paper chromatography, the only sugars present were glucose and mannose (solvent a).

Purification of the Glucomannan

Polysaccharide (IVa) was suspended in dimethylsulfoxide (800 ml) which was stirred vigorously for three hours and allowed to stand overnight. The insoluble part was removed by centrifugation and treated again with dimethylsulfoxide as described above. The light brown insoluble part was centrifuged, washed with this reagent, resuspended in methanol, filtered through cloth, washed with methanol and dried, yield 24 g (IV). The glucomannan (IV) indicated a specific optical rotation of $[\alpha]_D^{18}$ $-39°$ (10% NaOH, c, 0.9) and contained mannose and glucose in the ratio 3.6 : 1, together with a trace amount of xylose and galactose. Dimethylsulfoxide-soluble part (B) produced a bulky precipitate by pouring into methanol. This was separated as usual manner and the resultant white powder (9.8 g) containing xylose, arabinose and 4-O-methylglucuronic acid as component sugars, showed $[\alpha]_D^{15}$ $-49°$ (1% NaOH, c, 1.0).

Acetylation of the Glucomannan

The glucomannan (IV) (9.0 g) was dissolved in warm water (2 l, 70°C) with stirring and standing for 2 days at 0°, the insoluble material being separated by centrifugation. The centrifugate was concentrated in vacuo and poured into 5 volumes of methanol. The white precipitate separated was resuspended in methanol, filtered through cloth, and methanol was removed as much as possible by evaporation under reduced pressure. The methanol-wet glucomannan was dispersed in dry pyridine (100 ml) and then there was added dropwise 130 ml of acetic anhydride with vigorous stirring and ice-cooling. The temperature of the reaction mixture was maintained to 30° during this time, after which stirring was continued at room temperature for twenty-four hours. Portions of pyridine and acetic anhydride were added over five days until a total of 210 ml of pyridine and 290 ml of acetic anhydride had been added. After addition of these reagent, the temperature of the liquid was raised to 40° which was maintained for twenty-four hours with continuous stirring. The resultant light brown solution was poured into ice-water (3 l), whereupon the acetylated glucomannan appeared as a flocculent precipitate. The suspension was thoroughly extracted with chloroform but a large portion of the glucomannan acetate formed emulsion, which was extracted with chloroform several times, the clear chloroform extracts being combined and used for the preparation of fraction (GM I) and (X I). Partly acetylated glucomannan (Ac, 30.5%, 11 g) was recovered from the emulsion and acetylated again using 130 ml of dry pyridine and 113 ml of acetic anhydride. The reaction was complete for three hours at 40° and then the resultant brown solution poured into ice-water containing hydrochloric acid (30 g). The fibrous precipitate produced was filtered, washed with water and dried over anhydrous calcium chloride and then sodium hydroxide, yield 11.5 g, Ac, 43.9%.

Fractional Precipitation of the Glucomannan Acetate

The acetate (4.0 g) was dissolved in chloroform (300 ml) and precipitated in the usual way by the gradual addition of equal volume of petroleum ether (b.p. 30~50°). The precipitate was dissolved in chloroform and precipitated again as a white powder by pouring this solution into 10 volumes of petroleum ether. It was recovered and divided into three portions by fractional precipitation of its chloroform solution, each portions being refractionated further into two or three. The individual fractions were treated as described above, and the resultant white powders washed with petroleum ether and dried in vacuo. A soluble part in chloroform-petroleum ether (1 : 1) was made precipitate by concentration and addition of petroleum ether and the last fraction obtained. The specific optical rotation and sugar composition of each fraction indicate that fraction (22) to (32) inclusive are essentially homogeneous as given in Table I. They were combined and characterized, $[\alpha]_D^{20}$ $-30.7°$ (chloroform, c, 1.0). *Anal.* Found: $COCH_3$, 44.5. Calcd. for $C_{12}H_{16}O_8$: $COCH_3$, 44.8%.

Deacetylation of the Glucomannan Triacetate

To a solution of the glucomannan triacetate (180 mg) in chloroform (18 ml), 0.2 N sodium methoxide in abs. methanol (9 ml) was added with shaking and the mixture was allowed to stand over-night at room temperature. After addition of methanol (27 ml), acetic acid and water, the resultant white precipitate was removed by filtration and washed with 75% ethanol, ethanol and dried, yield 90 mg, $[\alpha]_D^{22}$ $-36.5°$ (10% NaOH, c, 1.3). Determination of mannose content in the glucomannan was carried out as described in the previous report[15] which was modified the Hägglund's method[16]. To make complete the precipitation of mannose phenylhydrazone, however, the hydrolyzed solution was kept to fourty-eight hours

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[15] T. Koshijima and I. Tachi, This Bulletion, **21**, 253 (1957).

[16] E. Hägglund and L. C. Bratt, *Sevensk Kem. Tidskr.*, **48**, 125 (1936).

TABLE I. FRACTIONAL PRECIPITATION OF THE GLUCOMANNAN ACETATE

Fraction	Acetyl, %	Yield, %[a]	$[\alpha]_D$ in Chloroform	Xylose	Mannose	Glucose	Galactose
1	43.2	46.3	—	0.11	2.5	1	0.15
11		22.1	—	0.07	1.4	1	0.12
12		11.8	−21.8	0.03	1.0	1	0.13
13		3.8	−19.4	Trace	1.4	1	Trace
2	44.8	27.5	−30.0	Trace	3.6	1	None
21		1.8	−44.5	Trace	4.6	1	None
22		14.0	−30.6	None	3.7	1	None
23		6.8	−30.8	None	3.5	1	None
3	44.3	25.8	−30.2	None	3.6	1	None
31		18.2	−30.7	None	3.6	1	None
32		3.2	−30.6	None	3.7	1	None
4		0.5	−20.5	None	2.1	1	None

a) Expressed on the original acetate.

TABLE II. FRACTIONATION OF THE GLUCOMANNAN ACETATE BY JONES' PROCEDURE

Fraction	Acetyl, %	Yield, %[a]	$[\alpha]_D$ in Chloroform	Xylose	Mannose	Glucose	Galactose
GMI	44.2	0.5	−30.8	Trace	3.5	1	0.03
GMII	44.5	31.3	−30.6	None	3.6	1	0.03
XI		0.8	−41.0	0.23	2.7	1	0.20
XII		28.0	—	—	—	—	—
E1		5.0	−33.7	0.20	2.1	1	0.05
2		11.0	−31.1	0.03	3.6	1	None
3		3.0	−28.6	Trace	3.4	1	None
4		Trace	—	Trace	2.0	1	None
R		9.1	—	0.20	1.4	1	0.06

a) Expressed on the original acetate.

at 0° and washings of the precipitate were effected successively by two of 1 ml of cold water, two of 1 ml of cold ethanol and 2 ml of ether for 224 mg of the hydrazone. The hydrazone derived from the glucomannan (67.0 mg) was 82.0 mg: this corresponds to 78.5% mannose residue.

Fractionation of Glucomannan Acetate by the Jones' Procedure

To the chloroform solution of the glucomannan acetate (3.5 g in 500 ml), saturated sodium bicarbonate solution was added and shaken. The chloroform layer which separated was removed and again treated with this reagent further two times. It was combined to the washings of emulsion, washed with water, dried over anhydrous sodium sulfate, concentrated to 150 ml and poured into petroleum ether. The glucomannan triacetate (GM II) was recovered as white yellow powder, yield 0.94 g $[\alpha]_D^{18}$ −30.6° (chloroform, c, 1.5).

The emulsion was washed with chloroform, acidified with ice-cold 1 N hydrochloric acid and shaken with chloroform, whereupon chloroform layer did not separate from the emulsion. Equal volume of water was added and chloroform layer separated. The dried chloroform solution (X II) was fractionally precipitated as before and fraction (E1) to (E4) obtained. The emulsion remaining after the repeated extraction with chloroform was recovered by pouring into methanol (ER). The partly acetylated glucomannan was treated in the same way as described here and small amount of glucomannan triacetate (GM I) was obtained, $[\alpha]_D^{18}$ −30.8° (chloroform, c, 1.0), as well as fraction (X I) (see Table II).

Determination of Intrinsic Viscosity

The intrinsic viscosity was measured on the chloroform solution of fraction (22) and (31) at 20±0.01° using an Ubbelohde capillary viscometer. In each of the cases, the reduced viscosity was determined at 3 different concentrations and linearly extrapolated to zero concentration. Fraction (22), $\eta_{sp}/c.$, 1.141, 1.048, 0.961; $[\eta]$, 0.776.

Fraction (31), $\eta_{sp}/c.$, 0.538, 0.478, 0.420; $[\eta]$, 0.291.

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Studies on the Conjugated Lipids

Part XIV*. Fatty Acid Constituents of Sphingomyelin from Horse Spinal Cord**

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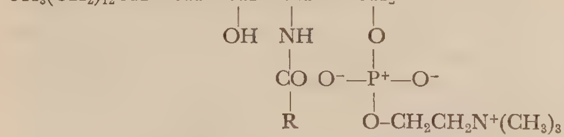
Received June 9, 1960

The chemical nature of fatty acids was studied on sphingomyelin which was prepared from horse spinal cord. Nervonic, palmitic, stearic and behenic acids were isolated from the hydrolysate of the sphingomyelin preparation. The presence of behenic acid in sphingomyelin has in this paper been reported for the first time.

It is known that several kinds of fatty acid can attach to the amino group of the shingosine moiety of shingomyelin, the structure of which is shown in Fig. 1. However, an assortment of fatty acids in shingomyelin from a certain animal tissue seems to be almost settled, althouh some confusion and inconsistencies on this point are seen in the literature. This may be unavoidable because sphingomyelin prepared by the older methods might often have been contaminated with other lipids. Nowadays, it is generally believed that sphingomyelin from brain contains lignoceric, stearic and nervonic acids as the component fatty acids$^{1\sim3)}$, and that sphingomyelin from lung or spleen contains lignoceric and palmitic acids$^{3,4)}$. The present authors undertook to elucidate the fatty acid constituents of sphingomyelin from spinal cord, since the tissue has never been studied in respect to this problem.



$$CH_3(CH_2)_{12}CH=CH-\underset{\substack{|\\ OH}}{CH}-\underset{\substack{|\\ NH\\ |\\ CO\\ |\\ R}}{CH}-\underset{\substack{|\\ O\\ |\\ O^- - P^+ - O^-\\ |\\ O-CH_2CH_2N^+(CH_3)_3}}{CH_2}$$

R: Fatty alkyl group

FIG. 1. Structure of Sphingomyelin

A pure sample of sphingomyelin was prepared from horse spinal cord, and hydrolyzed with methanolic sulfuric acid to produce methyl esters of fatty acids, which were divided into three fractions, 1, 2 and 3, according to their respective solubilities. Free fatty acids were isolated by saponification of each fraction, and then subjected to investigations of their physical and chemical properties.

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* Part XIII: This Bulletin, **22**, 426 (1956).

** A preliminary communication of this work has been published in the *Nature*, **184**, 817 (1959).

1) P. A. Levene, *J. Biol. Chem.*, **24**, 69 (1916).

2) W. Merz, *Z. Physiol. Chem.*, **193**, 59 (1930).

3) S. J. Thannhauser and N. F. Boncoddo, *J. Biol. Chem.*, **172**, 141 (1948).

4) S. J. Thannhauser, J. Benotti and N. F. Boncoddo, *J. Biol. Chem.*, **166**, 677 (1946).

Fatty acid from fraction 1 was proved to be nervonic acid on the basis of melting point, elementary analysis, molecular weight by titration and iodine number. Fatty acid from fraction 2 was found to consist of equal portions of palmitic and stearic acids judging from neutralization equivalent, chromatographic behavior and melting point. Fatty acid from fraction 3 was at first anticipated to be lignoceric acid, the predominant acid in the usual preparations of sphingomyelin, since this fraction yielded a larger amount of acid than the other fractions. The melting point of the material and its methyl ester, however, did not agree with that of lignoceric, but with that of behenic acid series. The neutralization equivalent, paper chromatography and the infra-red spectra indicated with certainty that the fatty acid from fraction 3 was behenic acid.

It has been shown, therefore, that sphingomyelin from horse spinal cord contain nervonic, palmitic, stearic and behenic acids as the fatty acid constituents, although the possibility is not completely excluded that some other acids may as well exist in small quantities in the preparation. Behenic acid among the four mentioned above is the one which has never been described in the literature as the principal component acid in sphingomyelin from normal animal tissue, though it has been detected in cerebroside from spleen of Gaucher's disease5,6) as well as in sphingomyelin from brain in a very small amount7).

EXPERIMENTAL

1. Preparation of Sphingomyelin*

Crude sphingomyelin prepared from the lipid fraction of horse spinal cord was carefully purified by treatment with alkali4) and successively by alumina column chromatography8). The purified sphingomyelin was several times recrystallized from ethyl acetate. Thus, 3 kg of fresh tissue yielded at most about 15 g of snow-white crystals of sphingomyelin. M.p. 197～198°C. $[\alpha]_D^{20}=+5.2°$(c=0.97 in chloroform-methanol). The preparation was practically pure as judged from the analytical data, being quite uncontaminated with glycerolipids or glycolipids. *Anal.* Found: P, 3.95; N, 3.46; P:N=1:2; glycerol, negligible; sugar, trace. Calcd. for $C_{45}H_{93}N_2PO_7$ (behenyl-sphingomyelin): P, 3.85; N, 3.47; P:N=1.0:2.0; glycerol, zero; sugar, zero.

2. Hydrolysis of Sphingomyelin

Six grams of pure sphingomyelin were refluxed for eight hours with 200 ml of 10% sulfuric acid in methanol. The reaction mixture was allowed to stand in an ice box overnight after hydrolysis. Large amounts of the precipitate formed on cooling were separated from the mother liquor by filtration and washed with cold methanol. The filtrate was shaken several times in a separatory funnel each time with 80 ml of petroleum ether to romove methyl esters of unsaturated fatty acids. The petroleum ether fraction was twice washed with water and concentrated to dryness by distilling under reduced pressure; weight 0.5 g (fraction 1). The precipitate, chiefly methyl esters of saturated acids, consisted of a smaller amount of yellow semisolid lump and a larger quantity of white solid material. The former was simply separated from the latter by picking up with a pincette, washed once with methanol and dried; weight 0.7 g (fraction 2). The solid material was brought to dryness as it was, to yield a rustling powder; weight 1.4 g (fraction 3).

3. Isolation of Fatty Acids

Each fraction obtained above was saponified for one hour with 30 volumes of 0.5 N potassium hydroxide in methanol. An equal volume of ether was added to the reaction mixture to precipitate the salts of fatty acids, which formed a gel on cooling. After having been filtered and washed with ether, the potassium salts were dissolved in water, removed to a separatory funnel, acidified with 2 N sulfuric acid and then shaken with ether. The ether layer was washed with water, kept with anhydrous sodium sulfate overnight and evaporated under diminished pressure. The residue obtained was recrystallized repeatedly to a constant melting point by dissolving in boiling absolute ethanol and subsequent

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* Details will be published in another communication.

5) E. Klenk, *Z. Physiol. Chem.*, **267**, 128 (1940～41).

6) A. Rosenberg and E. Chargaff, *J. Biol. Chem.*, **233**, 1323 (1958).

7) F. Rennkamp, *Z. Physiol. Chem.*, **284**, 215 (1949).

8) E. Klenk and F. Rennkamp, *Z. Physiol. Chem.*, **267**, 145 (1940～41).

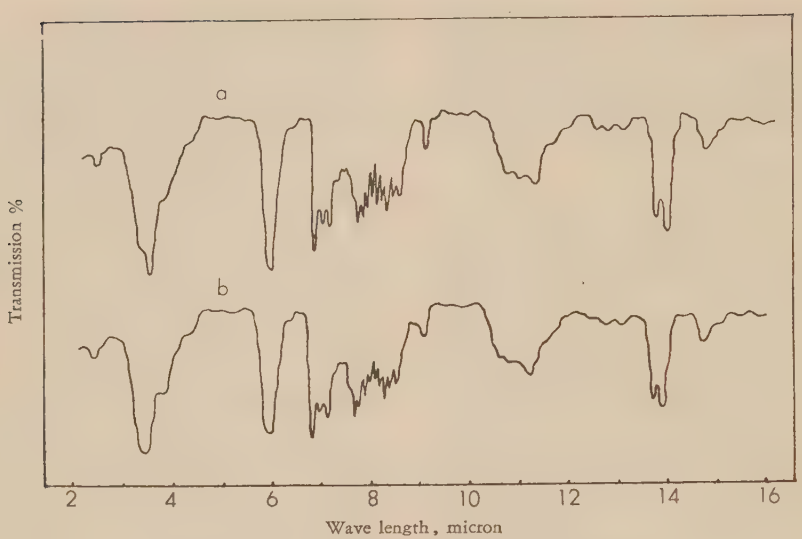

FIG. 2. Infra-red Spectra of Behenic Acid (KBr).
a: Authentic behenic Acid
b: The acid from fraction 3

cooling in a refrigerator. Thus, fraction 1 yielded 0.1 g, fraction 20.6 g and fraction 30.9 g of free fatty acid, respectively, as a white crystalline material.

4. Identification of Fatty Acid from Fraction 1

Fatty acid from fraction 1 melted at 39~40°, which suggested its possible identity with nervonic acid, m.p. 41°C. The analytical data, molecular weight by titration and iodine number were in good agreement with those of nervonic acid. *Anal.* Found: C, 78.11; H, 12.32; M.W. 360; iodine number, 65. Calcd. for $C_{24}H_{46}O_2$ (nervonic acid): C, 78.26; H, 12.56; M.W., 366; iodine number, 69.3.

5. Identification of Fatty Acid from Fraction 2

Fatty acid from fraction 2 melted at 54~55°C which apparently suggested a relationship with myristic acid, but the neutralization equivalent made if necessary to consider it a mixture of palmitic and stearic acids. The paper chromatogram[9] supported the latter suggestion; two spots on the paper clearly corresponded to the standard samples of palmitic and stearic acids, respectively. In addition, the mixture of authentic palmitic and stearic acids (1 : 1) gave a melting point of 55°C. These results indicate that the material from fraction 2 consists of equal parts of palmitic and stearic acids, although the analytical value of carbon in the elementary analysis was somewhat out of line. *Anal.* Found: C, 77.56: H, 12.60; M.W., 270; iodine number, trace. Calcd. for $C_{16}H_{32}O_2$ (palmitic acid): C, 75.18; H, 12.48; M.W. 256, iodine number, zero. Calcd. for $C_{18}H_{36}O_2$ (stearic acid): C, 76.05; H, 12.67;

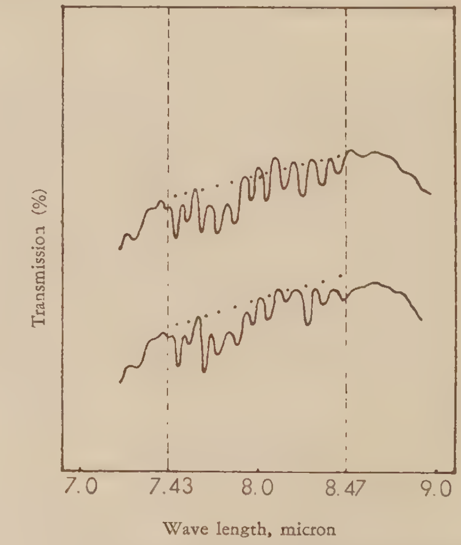

FIG. 3. Infra-red Spectra of Barium Behenate (KBr).
a: Authentic barium behenate
b: Barium salt of the acid from fraction 3
Dot: Band number

M.W., 284; iodine number, zero.

6. Identification of Fatty Acid from Fraction 3

Fatty acid from fraction 3 could not be identified from the elementary analysis. However, the melting point of the material, 79~80°C, and that of its methyl ester, 52.5~53.5°C, suggested it to be behenic acid. The neutralization equivalent and paper chromatography[9] supported this. *Anal.* Found: C, 76.95, H, 12.84; M.W., 342; iodine number, trace. Calcd. for $C_{22}H_{44}O_2$ (behenic acid): C, 77.58; H, 13.02; M.W., 340; iodine number, zero.

Furthermore, the infra-red spectra of this material and its barium salt were identical with those of C_{22} series, as shown in Fig. 2 and Fig. 3. It was especially striking that in the spectrum of the salt in Fig. 3 eleven bands were distinctly recognized between wave lengths 7.43 and 8.47 μ, which are characteristic of the salt of a C_{22} acid since the number of bands in this region is equal to half the number of carbons in the straight chain-fatty acids according to Meiklejohn et al.[10]. Thus, it is certain that the fatty acid from fraction 3 is behenic acid.

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[9] Y. Inouye and M. Noda, *J. Agr. Chem. Soc. Japan*, **26**, 634 (1952).

[10] R. A. Meiklejohn, R. J. Meyer, S. M. Aronovic, H. A. Schuette and V. W. Meloch, *Anal. Chem.*, **29**, 329 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol, 24, No. 7, p. 695~698, 1960]

The Studies on the Action of Yeast Hexokinase upon the α- and β-Diasteromers of D-Glucose

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The yeast hexokinase is highly specific for α-isomer of D-glucose. The relative rate of phosphorylation of β-D-glucose, catalyzed by the purified yeast hexokinase, is observed to be 60~70 (α-D-glucose=100). The average Michaelis constants of yeast hexokinase are found to be 1.8×10^{-4} and 2.4×10^{-4} for α-D-glucose and β-D-glucose respectively, therefore the difference between the two constants is considered to be negligible.

INTRODUCTION

Although fairly stable in crystalline form, sugars undergo many transformations when dissolved in water. The interconversion between the α- and β-diasteromers of the same enatiomorph takes place under the mildest possible condition. This phenomenon was first observed by Dubrunfaut[1] who noted that the optical rotation of freshly dissolved glucose changed with time and that after a number of hours, the rotation became constant. For example, the freshly prepared aqueous solution of the α-isomer of D-glucose mutarotates downward, and that of the β-isomer mutarotates upwards; in both cases the same equilibrium value is reached.

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[1] cf. W. W. Pigman, "Chemistry of the Carbohydrate", Academic Press, 1948, p. 63.

It is well known that some of the purified enzymes do not act, with the same rate, upon both D- and L-enatiostereomers which are fairly stable in water. It is also known that the purified glucose oxidases, of mold[2] and of mammalian liver[3], are highly specific for β-D-glucose.

But no worker described about the effects of the purified yeast hexokinase upon the α- and β-D-glucose, which undergo considerably rapid mutarotation when dissolved in water. Gottschalk reported that the α- and β-isomers of D-glucose[4], and those of D-mannose[5], were fermented well with yeast cells, but no experiment was carried out by using the purified enzyme preparation. This raised the question whether the purified yeast hexokinases catalyzes the phosphorylation of both α- and β-isomers of D-glucose with the equal rate.

METHODS

1. Preparation of α-D-glucose

100 g of D-glucose was dissolved in 100 ml of water at 0°C, and the insoluble part (the α-isomer) was obtained by filtration. Its m.p. 146°C; $[\alpha]_{\mathrm{D}}^{20}=+112.2° \rightarrow +52.7$ (with 4% aqueous solution).

2. Preparation of β-D-glucose[6]

The β-isomer was prepared by crystallization from hot acetic acid at a temperature higher than 98°C and recrystallization from water and alcohol at a lower temperature. Its m.p. 148～150°C; $[\alpha]_{\mathrm{D}}^{20}=+18.7 \rightarrow +52.7$ (with 4% aqueous solution).

3. Determination of the proportion of the α- and β-isomers

a). Calculation from the observed rotation. The specific rotation ($[\alpha]_{\mathrm{D}}^{20}$) of freshly dissolved α-D-glucose, freshly dissolved β-isomer and of the equilibrium solution are +112.2°, +18.7° and +52.7° respectively. The proportion of the α- and β-isomers of D-glucose in the equilibrium aqueous solution is known to be 37 : 63. Therefore, the proportion of the α- and β-isomers in the sample can be calculated from the observed rotation and the concentration of the sample.

b). The oxidation method. The proportion of the α- and β-isomers of D-glucose can be determined also by the modified oxidation method[7,8]. The assay method is as follows: An aliquot of the sample is diluted to 1% glucose concentration with 0.1 M-phosphate buffer (pH 5.7). 2 ml of the diluted sample is withdrawn using a pre-cooled pipette, and is added to 10 ml of the oxidizing mixture (0.4 M-phosphate buffer of pH 5.7, containing bromine; 40% saturation) in a stoppered tube, pre-cooled to 0°C in an ice bath. After mixing, the solution is kept at 0°C for exactly eight minutes. The excess bromine is removed by addition of 1 ml of a mixture of corn oil and chloroform (1 : 1) followed by vigorous shaking. The resulting emulsion is centrifuged and the clear supernatant containing unoxidized glucose is diluted (usually by a factor of 25) and assayed for glucose. Under these conditions, the amount of β-D-glucose oxidized is about 84% and that of α-D-glucose is 14%. Therefore the proportion of the α- and β-isomer is calculated by the following relations:

$$a=(10d-1.6c)/7\ ;\quad b=(8.6c-10d)/7$$

where a is the amount of α-D-glucose in the sample, b is that of the β-isomer, c is the total amount of D-glucose in the sample before oxidation, and d is the amount after oxidation.

4. Preparation of yeast hexokinase.

The yeast hexokinase was purified in accordance with the method of Kunitz and McDonald[9], though it was not crystallized. The specific activity of the preparation was about 550 units per mg of protein.

5. Assay method.

Hexokinase (about 2 units) is added to the vessels, each of which contains 1.0 ml of 2.0 M-Tris buffer (pH 7.0), 0.2 ml of 0.2 M-magnesium chloride, 0.5 ml of 0.01 M-D-glucose (the freshly prepared aqueous solution of α-isomer, or of β-isomer, or the equilibrium solution), 0.4 ml of 0.5 M-sodium fluoride, and water. Each 3.0 ml of each reaction mixture (6.0 ml) is immediately withdrawn using a pipette, and is added to 3.0 ml of 0.3 N-barium hydroxide. Each reaction mixture is incubated for a certain minutes at 30°C, and is added to 3.0 ml of 0.3 N-barium hydroxide. After the addition of each 3.0 ml of 5% zinc sulfate to each reaction mixture, each one is filtered to remove protein and D-glucose-6-phosphate. The amount of D-glucose in each mixture is determined by the method of Somogyi and Nelson[10].

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[2] D. Keilin and E. F. Hartree, *Biochem. J.*, **50**, 331 (1952).

[3] H. J. Strecker and S. Korkes, *J. Biol. Chem.*, **196**, 769 (1952).

[4] A. Gottschalk, *Australian J. Exp. Biol. and Med. Sci.*, **21**, 133 (1943); **22**, 291 (1944).

[5] A. Gottschalk, *Biochem. J.*, **41**, 276 (1947).

[6] C. S. Hudson and J. K. Dale, *J. Am. Chem. Soc.*, **39**, 323 (1917).

[7] D. S. Bhate and R. Bentley, *Federation Proc.*, **16**, 154 (1957).

[8] R. Bentley, Personal communication, July 10 (1959).

[9] M. Kunitz and M. R. McDonald, *J. Gen. Physiol.*, **29**, 393 (1946).

6. Definition of unit.

One unit of hexokinase is defined as the amount of enzyme which catalyzes the phosphorylation of 1 μmole of α-D-glucose in fifteen minutes at 30°C in the above mentioned condition. Specific activity is expressed as hexokinase units per milligram of protein. Protein is determined by the method of Johnson[11].

RESULTS

1. The mutarotation coefficient of the α- and β-isomer in the aqueous solution. Isbell and Pigman[12] demonstrated that the mutarotation coefficients of α- and β-isomers of D-glucose, k_1+k_2, where k_1 is the reaction constant for $\alpha\rightarrow\beta$, and k_2 is the same for $\beta\rightarrow\alpha$, are 0.00632 and 0.00625 respectively (at 20°C). Their values are almost solid for the authors' results, 0.0065 at pH 7.0 at 30°C, which are shown in Fig. 1.

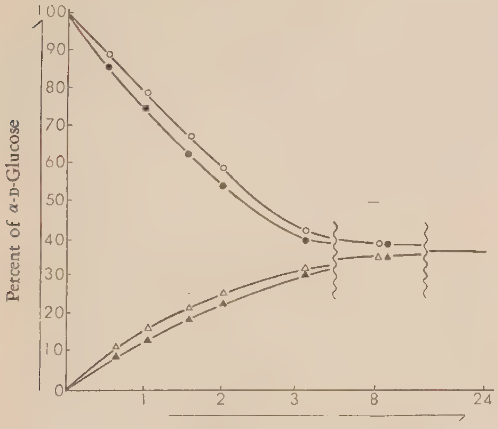

FIG. 1. The Mutarotation of the α- and β-D-Glucose in the Aqueous Solution (pH 7.0) at 30°C.

The experiments were carried out with the freshly dissolved 1% aqueous solution of α-D-glucose (○, ●) and that of β-D-glucose (△, ▲). ○, △: Determined by observed rotation. ●, ▲: Determined by oxidation method.

2. The effect of the crude yeast hexokinase (fraction 0.70 of Kunitz and McDonald[9]) upon the α- and β-isomers of D-glucose. The representative results are shown in Fig. 2. The difference between the velocity of the phosphorylation of the α- and β-isomers of D-glucose, catalyzed by the crude yeast hexokinase, is observed to be negligible.

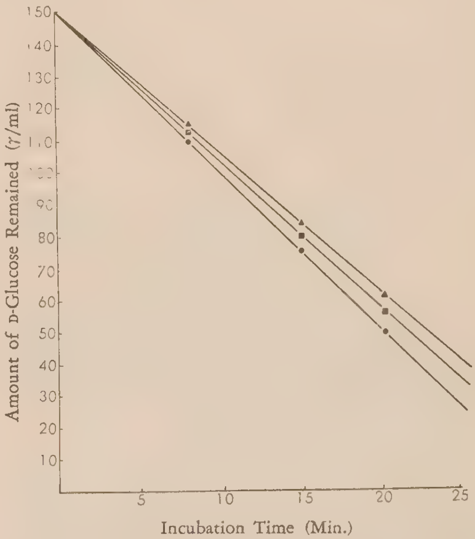

FIG. 2. The Effect of the Crude Yeast Hexokinase upon the α- and β-D-Glucose.

The crude yeast hexokinase was added to the vessels, each of which contains 10 ml of 0.2 M-Tris buffer (pH 7.0), 2 ml of 0.2 M-$MgCl_2$, 5 ml of freshly dissolved 0.01 M-D-glucose (in one case the α-isomer ●, and in the other the β-isomer ▲), or 5ml of the 0.01 M-equilibrium aqueous solution of D-glucose ■, 4 ml of 0.5 M-sodium fluoride, and water to 60 ml. The reaction mixture was incubated at 30°C.

3. The effect of the purified yeast hexokinase upon the α- and β-isomers of D-glucose. The representative results are shown in Fig. 3. The velocity of the phosphorylation of the α-D-glucose, catalyzed by the purified yeast hexokinase, is observed to be greater than that of the β-isomer.

4. Kinetics. The representative results are shown in Fig. 4. Michaelis constant of the enzyme upon α-D-glucose is observed to be 1.8×10^{-4}, and that of the same enzyme upon the β-isomer is observed to be 2.4×10^{-4}.

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[10] M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945); M. Somogyi, *ibid.*, **160**, 74 (1945).

[11] M. J. Johnson, *J. Biol. Chem.*, **137**, 575 (1941).

[12] H. S. Isbell, W. W. Pigman, *J. Research Natl. Bur. Standards*, **18**, 141 (1937).

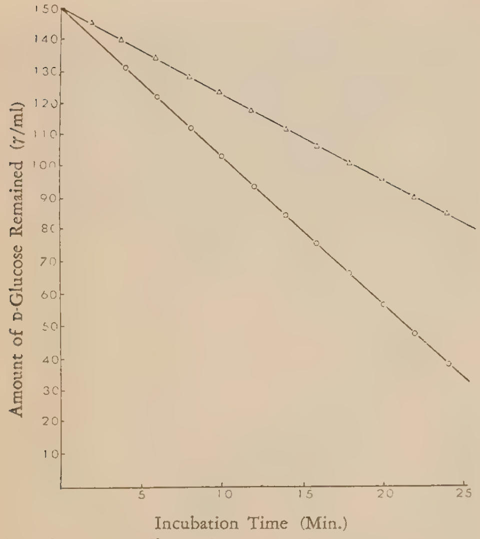

FIG. 3. The Effect of the Purified Yeast Hexokinase upon the α- and β-D-Glucose.

The purified yeast hexokinase was added to the vessels, each of which contains 30 ml of 0.2 M-Tris buffer (pH 7.0), 6 ml of 0.2 M-$MgCl_2$, 15 ml of freshly dissolved 0.01 M-D-glucose (in one case the α-isomer ○), and in the other case the β-isomer △), 12 ml of 0.5 M-sodium fluoride, and water to 180 ml. The reaction mixture was incubated at 30°C.

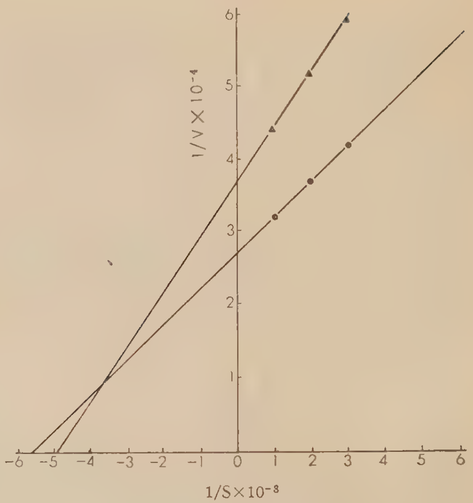

FIG. 4. Comparison of Rate of Phosphorylation of Freshly Dissolved α-D-Glucose (●) with that of β-D-Glucose (▲).

S, the substrate concentration, is given in moles per liter. V, the volocity of the reaction, is given in moles of phosphorylated glucose per liter per minute. 1 unit of the purified enzyme is used per 1 ml of the reaction mixture.

SUMMARY

(1) The velocity of the phosphorylation of α-D-glucose, catalyzed by the purified yeast hexokinase, seems to be greater than that of β-D-glucose. The relative rate of phosphorylation of β-D-glucose is observed to be 60~70 (α-D-glucose=100).

(2) The average Michaelis constants of yeast hexokinase are found to be 1.8×10^{-4} and 2.4×10^{-4} when it acted on α-D-glucose and β-D-glucose respectively.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 699~702, 1960]

Inhibition Pattern of Beet-Saponin on Amylose Formation by Potato Phosphorylase

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Received May 30, 1960

By using the multiple ascent technique, the authors have resolved the first several oligosaccharides of the product of initial stage of potato phosphorylase action in both the absence and the presence of beet-saponin with maltotriose as primer. The resolved chromatogram was sprayed with a mixture of G-1-P and phosphorylase, followed by spraying with iodine solution to locate the spots in which starch synthesis occurred.

Multi-chain mechanism of amylose formation from maltotriose and the suppression of lengthening of amylose chain by beet-saponin in the lag stage of enzyme action could be shown on paper chromatogram. No saccharides other than amylose series were recognized in the case of phosphorylase inhibition by beet-saponin.

INTRODUCTION

It has been revealed that sugar beet and mangold contain natural inhibitors of phosphorylase[1]. Isolation and identification of the inhibitor from beet juice had been carried out by the authors in a large scale[2,3] and lead to the conclusion that a triterpenoid saponin, consisting of oleanolic acid, glucose and arabinose, is the principle of the inhibiting action[4,5]. The mode of action of several inhibitors of phosphorylase has never been studied from the standpoint of the character of the product.

In the synthesis of starch from α-D-glucose-1-phosphate (G-1-P) catalyzed by potato phosphorylase, an acceptor which is sometimes called activator or "primer molecule" is required to transfer the glucose units from G-1-P to the non-reducing end of an already existing polysaccharide chain.

Small amount of dextrin or soluble starch has been used as the source of primer. However, it is unsatisfactory to study the enzyme product by paper chromatography. Whelan and Bailey[6] demonstrated that maltotriose is the smallest molecule which will function as primer. They concluded[7] that the action pattern of potato phosphorylase is completely multi-chain, in which the random addition of the glucose units occurs and all the primer chains grow at approximately equal rates, from the results of an investigation of light absorption curves of iodine-stained products.

In this paper, the authors demonstrated the multi-chain mechanism on paper chromatograms and showed the suppression of lengthening of amylose chain by beet saponin in the initial stage of phosphorylase action with maltotriose as primer.

EXPERIMENTAL

The phosphorylase obtained from potatoes following the methods of Green and Stumpf[8] and Hidy and Day[9], was finally treated with a small amount of active charcoal (Takeda-Shirasagi) to eliminate the contaminant of priming substance and stored in the refrigerator at 5°C.

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[1] Y. Obata, M. Tomoeda and T. Yoshida, *J. Agr. Chem. Soc. Japan*, **28**, 132 (1954).

[2] Y. Obata, Y. Ishikawa and T. Yoshida, *ibid*. **29**, 947 (1955).

[3] Y. Obata, Y. Ishikawa and T. Yoshida, *ibid*. **30**, 89 (1956).

[4] Y. Obata, Y. Ishikawa and T. Yoshida *ibid*. **30**, 397 (1956).

[5] Y. Obata, and Y. Ishikawa **32**, 331 (1958).

[6] J. M. Bailey, W. J. Whelan and S. Peat, *J. Chem. Soc.*, **1950**, 3692.

[7] W. J. Whelan and J. M. Bailey, *Biochem. J.*, **58**, 560 (1954).

The primer-free dipotassium α-D-glucose-1-phosphate dihydrate was prepared by the method of McCready and Hassid[10] and was purified by ion exchange columns, Amberlite IRC-50 and Amberlite IR-4B. The phosphorus content of the crystals obtained was 8.22 per cent (Calcd. for $C_6H_{11}O_5OPO_3K_2 \cdot 2H_2O$: P, 8.33%).

Maltotriose was prepared from amylose obtained from potato starch following the method of Schoch[11]. Five g of 50% ethanol soluble oligosaccharide obtained from the partial acid hydrolysis of amylose was fractioned on a column of charcoal (100 g)—Celite 545 (100 g) and 0.2 g of chromatographically pure maltotriose was obtained by the method of Whelan, Bailey and Roberts[12].

A volume of 3 ml of phosphorylase reaction mixture contained 0.5 ml of the citrate buffer (0.5 M, pH 6.0), 0.2 ml of 1 per cent maltotriose solution, 0.3 ml of phosphorylase solution, 1 ml of water or 1 per cent beet saponin solution, and 1 ml of 0.1 M G-1-P solution. The G-1-P solution was added to the rest of the mixture after temperature equilibration.

Several vessels containing the phosphorylase reaction mixture with and without beet saponin were incubated at 20°C and portions were withdrawn at suitable time intervals for detection of product. The enzyme was inactivated by inserting the reaction vessels in a boiling water bath for five minutes.

Proper amounts of the reaction mixture were spotted at intervals of 2.5 to 3 cm along a line ruled 4 cm from the below end of a filter paper (Toyo Roshi No. 50). After drying, the paper was rolled into a cylinder and held in that form by a thread around it. The cylinder was placed in a shallow layer of solvent consisted of *n*-butanol, pyridine and water mixed in the ratio of 6 : 4 : 3 by volume, and developed upward at at room temperature.

After the solvent had reached as far as 30 cm from the starting line, the paper cylinder was removed from the tightly closed vessel, unrolled and air-dried. Five additional ascents were repeated for the better resolution of oligosaccharides[13].

Finally, 1 ml of phosphorylase solution and 1 ml of 0.1 M G-1-P and 8 ml of 0.05 M citrate buffer (pH 6.2) were mixed and immediately sprayed evenly on the resolved chromatogram. The damp paper was stored in a water-saturated cylinder at room temperature for several hours. After the paper was dried, it was sprayed with a 0.01 M iodine-potassium iodide solution to locate the areas in which starch synthesis had occurred[14].

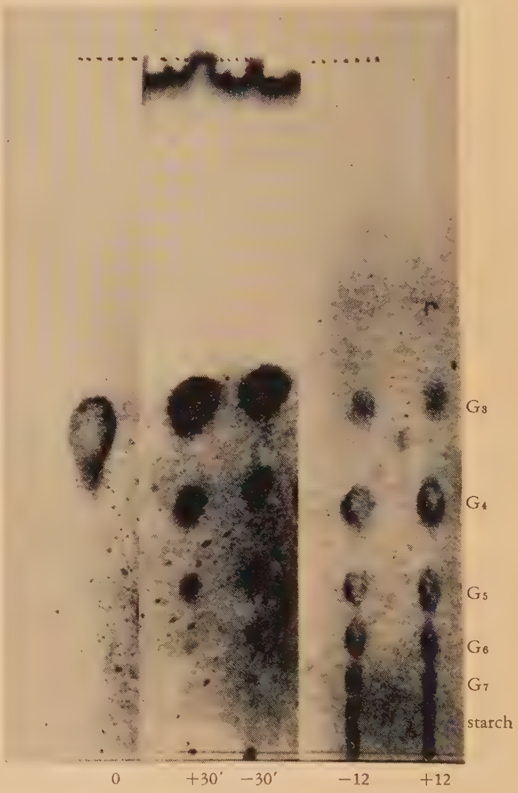

FIG. 1. Paper Chromatograms of Amylo-oligosaccharides Formed from Maltotriose Primer (0) by Potato Phosphorylase in Both the Absence (−) and the Presence (+) of Beet Saponin after 30 Minutes (30′) and 12 Hours (12) of Incubation at 20°C.

G_3, G_4, G_5, etc,, represent malto-triose, -tetraose, -pentaose, etc., after 6 multiple ascents with a solvent consisting of *n*-butanol, pyridine and water (6 : 4 : 3, v/v).

Phosphorylase reaction mixture: see the text.

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8) D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

9) P. H. Hidy and H. G. Day, *ibid.*, **160**, 273 (1945).

10) R. M. McCready and W. Z. Hassid, *J. Am. Chem. Soc.*, **66**, 560 (1944).

11) T. J. Schoch, *ibid.*, **64**, 2957 (1942).

12) W. J. Whelan, J. M. Bailey and P. J. P. Roberts, *J. Chem. Soc.*, **1953**, 1293.

13) A. Jeannes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

14) D. French and G. M. Wild, *J. Am. Chem. Soc.*, **75**, 4490 (1953).

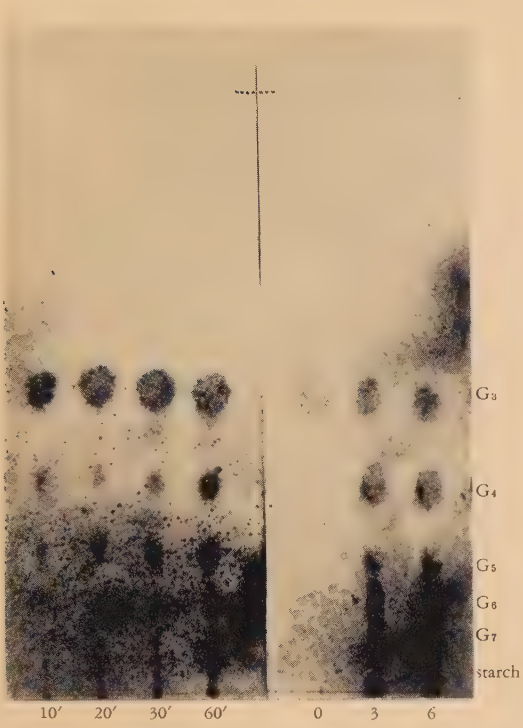

FIG. 2. Paper Chromatograms of Amylo-oligosaccharides Formed from Maltotriose Primer (0) by Potato Phosphorylase in the Presence of Beet Saponin after 10, 20, 30 and 60 Minutes (10′, 20′, 30′ and 60′), 3 and 6 Hours (3 and 6) of Incubation at 20°C.

G_3, G_4, G_5, etc., represent malto-triose, -tetraose, -pentaose, etc., after 6 multiple ascents with a solvent consisting of *n*-butanol, pyridine and water (6:4:3, v/v).

Phosphorylase reaction mixture: see the text.

RESULTS AND DISCUSSION

The results of two typical experiments on the effect of beet saponin on amylose chain formation are presented in Figs. 1 and 2.

Fig. 1 shows that after 30 minutes of incubation, the formation of a considerable amount of starch could be recognized as the spot on the starting line of the resolved chromatogram in the absence of saponin (−30′). And less amount of malto-pentaose and hexaose than those of amylo-oligosaccharide being formed in the presence of saponin (+30′) was detected. In contrast, starch formation was greatly suppressed in the presence of soponin (+30′). Only a trace of starch was found and a considerable amount of maltotetraose, -pentaose, -hexaose and even a trace of maltoheptaose were present indicating the inhibition of phosphorylase in the early stage of the development of amylose chain.

These results give support for the production of amylose chain, when maltotriose was used as primer, not by the single chain mechanism but by the multichain mechanism[7]. However, because of the much greater priming ability of the 4, 5, 6 and 7 glucose units compounds increasing in that order than that of 3 units, the very speedy extension in length of amylose chain to starch during the time interval before another maltotriose molecule is added by one glucose residue simulated the single chain pattern.

After 12 hours of incubation, paper chromatogram of the product (Fig. 1, −12 and +12) showed maltotetraose to be the major component with smaller amount of maltotriose, the primer used. All members of amylo-oligosaccharides were found and no apparent difference could be shown after 12 hours of enzyme action in both the presence (+12) and the absence (−12) of beet saponin.

In a preliminary examination, clear difference of the iodine stains between the product "−30′" (blue) and the product "+30′" (red), and the similarity of the iodine color reaction of those products "−12" (blue) and "+12" (blue) had been shown coinciding with the results represented in Fig. 1.

In Fig. 2, the products of phosphorylase action in the presence of beet saponin at the end of the incubation times of 10, 20, 30 and 60 minutes, 3 and 6 hours are presnted. The longer the time of enzyme action, the more and higher amylo-oligosaccharides appears in spite of the presence of beet saponin.

Another paper developed multiply just in the same way as described above and air-dried was sprayed with aniline hydrogen phthalate reagent or the locating reagent[15] with an alkaline copper reagent followed by a phosphomolybdic acid reagent to locate all sugars formed by the phosphorylase reaction with or without beet-saponin. However, no saccharide other than amylose series was detected on the paper chromatogram.

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[15] D. French, D. W. Knapp and J. H. Pazur, *J. Am. Chem. Soc.*, **72**, 5150 (1950).

Katagiri and Ikemiya[16] studied the inhibitory effects of synthetic detergents on purified α- and β-amylases from malt and reported that the anionic detergent is more inhibitory than the cationic. In an experiment of our laboratory, the strong inhibitory effect of beet saponin has been shown on β-amylase from rye.

Beet saponin seems to interfere in the series of binding positions of the enzyme which hold the substrate in appropriate steric relationship so that other functional groups present at the enzyme surface are able to complete the reaction and prevent a good fix of substrate with the enzyme, thus resulting in inhibition.

The authors wish to thank Mr. T. Yoshida, Takasago Perfumery Co., for his help in preparing maltotriose used in these experiments.

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16) H. Katagiri and M. Ikemiya, *Koso Kagaku Shinpojiumu*, **12**, 5 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 703~710, 1960]

The Amadori Rearrangement Product as an Intermediate in the Browning Reaction

Part I. Preparation and Infrared Spectra of 1-Amino-1-deoxy-D-fructoses and 1-Glycino-1-deoxy-D-tagatose*

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Received June 9, 1960

In relation to the elucidation of the mechanism of the browning reaction, isolation in crystalline form, structure and characterization of the intermediate compounds are studied. Glycine-isoglucosamine (1-deoxy-1-glycino-D-fructose) was prepared in crystalline form by conversions of glycine-N-D-glucoside and -N-D-mannoside (both Na salt) and also from heated solution of glycine and D-glucose. Similarly, crystalline glycine-isogalactosamine (1-deoxy-1-glycino-D-tagatose) (m.p. 165~168°C) was obtained by rearrangement of glycine-N-D-galactoside (Na salt) and from the browning mixture of glycine and D-galactose. Infrared studies indicated that the absorption near 3,500 cm^{-1} is characteristic to isoglucosamines but not to isogalactsamine. On the infrared and periodate oxidation data, the structure of the amino acid-isoglycosamines is considered.

The preparation of amino acid-isoglucosamine (1-amino-1-deoxy-D-fructose) or the Amadori rearrangement product of the reaction between an amino acid and D-glucose was first reported by Borsook and co-workers¹⁾, and its presumed significance in the biosynthesis of protein²⁾ was also suggested. The isolation from browned apricot purée of amino acid-isoglucosamine was described by Anet and Reynolds³⁾. Anet⁴⁾ reported the preparation of crystalline amino acid-deoxysugars with description of their color reactions, while Richards⁵⁾ isolated from the reaction mixture of glycine and glucose in the 'dry' state a crystalline reaction product which he considered to be a true intermediate in the browning reaction. The compound was shown to be the enolic form of N-(carboxymethyl)-1-amino-1-deoxy-fructose, and not the Amadori rearrangement product, the evidence of which was presented on the basis of the infrared spectra and other chemical properties.

It is of particular interest to note that the Amadori rearrangement product of arylamine has been shown by Micheel and Schleppinghoff⁶⁾ to give a characteristic absorption in infrared spectrum at 3570 cm^{-1}. Recently Heyns and Baltes⁷⁾ have reported that isoglucosaminuronic acid (1-amino-1-deoxy-D-fructuronic acid) shows no characteristic absorption band at 3570 cm^{-1} but similar band is absorbed at 3530 cm^{-1} by N-*p*-tolyl-isoglucosaminuronic acid. More recently, Heyns and Paulsen⁸⁾ presented the infrared spectra of amino acid-isoglucosamines, but they did not mention about the characteristic absorption band near 3500 cm^{-1}. It should also be noted that Micheel and Frowin[9] have reported that 4,6-substituted isoglucosamine shows no characteristic absorption band at 3570 cm^{-1}.

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* Presented in part at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 8, 1959.

1) A. Abrams, P. H. Lowy and H. Borsook, *J. Am. Chem. Soc.*, **77**, 4794 (1955); **78**, 3195 (1956).

2) H. Borsook, A. Abrams and P. H. Lowy, *J. Biol. Chem.*, **215**, 111 (1955).

3) E. F. L. J. Anet and T. M. Reynolds, *Nature*, **177**, 1082 (1956); *Austral. J. Chem.*, **10**, 182 (1957).

4) E. F. L. J. Anet, *Austral. J. Chem.*, **10**, 193 (1957).

5) E. L. Richards, *Biolchem. J.*, **64**, 639 (1956).

6) F. Micheel and B. Schleppinghoff, *Chem. Ber.*, **89**, 1702 (1956).

7) K. Heyns and W. Baltes, *Chem. Ber.*, **91**, 622 (1958).

8) K. Heyns and H. Paulsen, *Ann.*, **622**, 160 (1959)

The significance in the browning reaction of the Amadori rearrangement product has extensively been discussed by Hodge[10] and by other researchers[11], and there are a number of reports concerning the formation, synthesis and structure of the compound. Among them, Hodge[10] postulated a scheme for the browning reaction in which the Amadori rearrangement product plays an important role as an intermediate in the formation of the brown color. It is notable that in the definition of Hodge the Amadori rearrangement means the pathway from N-glycoside (glycosylamine) via 1,2-enolic form to 1-amino-1-deoxy-2-ketose.

Recently, Pigman and coworkers[12] have reported on the relationship between the Amadori rearrangement product of N-glycosylamine and the brown color formation in methanolic solution, and noted that N-*p*-tolyl-D-glucosylamine (GPT) and 1-deoxy-1-*p*-toluidino-D-fructose (DFT) behave differently in the color formation. Thus, they suggested that the acid-catalyzed destruction of DFT produced relatively little color but base-catalyzed destruction of DFT produced considerable amount of color, whereas acid-catalyzed destruction of GPT gave highly colored solutions, and that the transformation of DFT was essentially irreversible under the conditions cited.

It has long been supposed that in the Amadori rearrangement the reaction starts with the sugar-amine condensation product[10]. In dry media, the rearrangement was proved to start from the preformed N-substituted glycosylamines[13]. It was also reported that the N-substituted glycosylamines underwent the rearrangement by heating in alcoholic solution[14].

It is reasonable to assume that the Amadori rearrangement to produce amino acid-isoglucosamine starts from amino acid-sugar condensation product which is presumably N-glycoside of amino acid. However, no evidence has ever been presented to indicate that amino acid N-glucoside is really formed in the first step of the browning reaction in which the Amadori rearrangement is involved.

The present papar is concerned with the isolation of the Amadori products (glycine-isoglucosamine and glycine-isogalactosamine) in crystalline state from the rearrangement media of glycine-N-D-glucoside or glycine-N-D-mannoside and glycine-N-D-galactoside, respectively, as well as from the brown colored reaction mixtures from glycine and glucose or galactose. The infrared spectra of amino acid-N-glycosides and of isoglucosamine and isogalactosamine, in connection with the structure of them as the intermediate of the browning reaction, are also presented.

Amino acid-N-glycosides were synthesized by the procedures of Weitzel[15], and were subjected to the rearrangement in methanolic solution. Glycine-N-D-glucoside (Na salt) was heated in methanol for 2 to 4 hours and the reaction mixture was passed through a column of Dowex 50×4 (H$^+$ form) which was then eluted with 0.1 N ammonia. The fractions of the effluent which showed one spot of R_F 0.16 (ascending, BuOH-EtOH-water: 40-11-19) were collected and concentrated under reduced pressure. The syrup upon addition of methanol gave crystals melting at 156°C (dec.). Meanwhile, glycine and D-glucose (molar ratio 1 : 1 or 1 : 4) were allowed to react in an aqueous solution at 100°C for 1 hour and the reaction mixture was then treated by the procedure as described above. The fractions of the effluent showing the spot

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[9] F. Micheel and A. Frowin, *Chem. Ber.*, **92**, 304 (1959).

[10] J. H. Hodge, *Advances in Carbohydrate Chem.*, **10**, 169 (1955).

[11] J. P. Danehy and W. Pigman, *Advances in Food Research*, **3**, 241 (1951).

[12] L. Rosen, J. W. Woods and W. Pigman, *J. Am. Chem. Soc.*, **80**, 4697 (1958).

[13] Y. Inouye and K. Onodera, *J. Agr. Chem. Soc. Japan*, **22**, 120 (1948); J. E. Hodge and C. E. Rist, *J. Am. Chem. Soc.*, **75**, 316 (1953).

[14] R. Kuhn and A. Dansi, *Ber.*, **69**, 1745 (1936).

[15] G. Weitzel, H.-U. Geyer and A.-M. Fritzdorff, *Chem. Ber.* **90**, 1153 (1957).

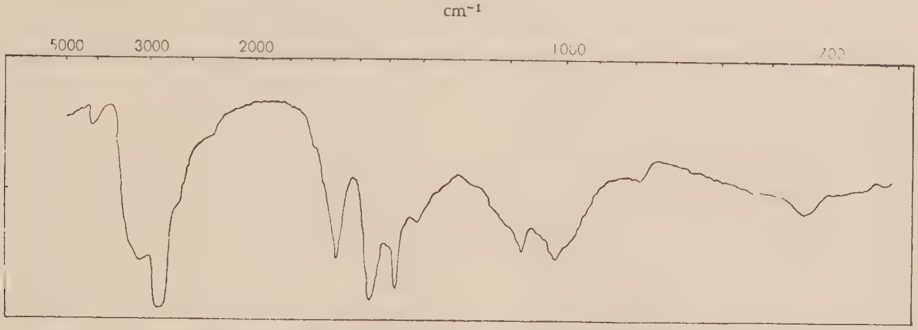

FIG. 1 a. Infrared Spectrum of Glycine-N-D-glucoside (Na salt) (Nujol).

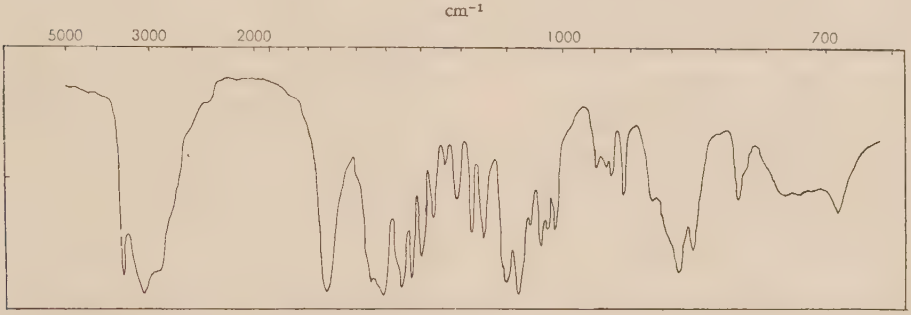

FIG. 1 b. Infrared Spectrum of 1-Deoxy-1-glycino-D-fructose (KBr).

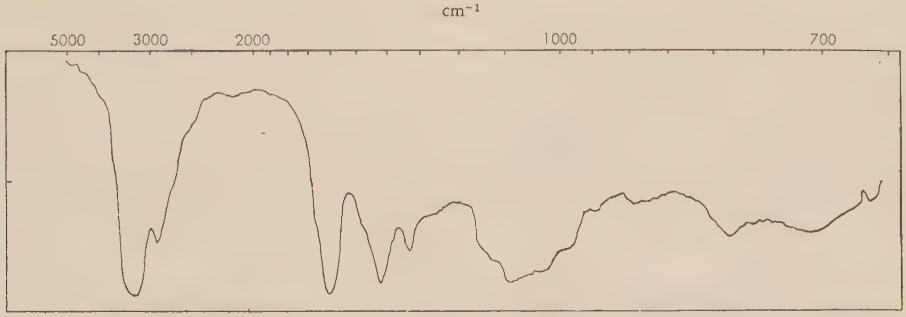

FIG. 1 c. Infrared Spectrum of Glycine-N-D-Galactoside (Na salt) (KBr).

of R_F 0.16 gave upon concentration the crystals of m.p. 156°C. The substance had the composition of $C_8H_{15}NO_7$, reduced methyleneblue in the presence of trace of alkali, and presented blue color with ferricyano ion. These color reactions are characteristic for isoglucosamine.

The same compound (m.p. 156°C) was obtained from the rearrangement medium of glycine-N-D-mannoside (Na salt). In this case the ammoniacal effluent of Dowex 50 column was submitted to the cellulose powder column chromatography.

There has been no report on the preparation of amino acid-isogalactosamine[16]. In this experiment glycine-isogalactosamine (1-deozy-1-glycino-D-tagatose) was obtained in crystalline

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16) Di-D-tagatose-glycine has been mentioned (E. F. L. J. Anet, *J. Am. Chem. Soc.*, **82**, 1502 (1960)).

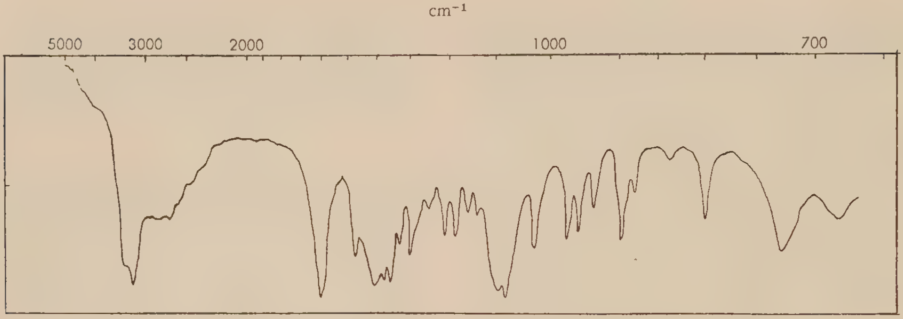


FIG. 1 d. Infrared Spctrum of 1-Deoxy-1-glycino-D-tagatose (KBr).

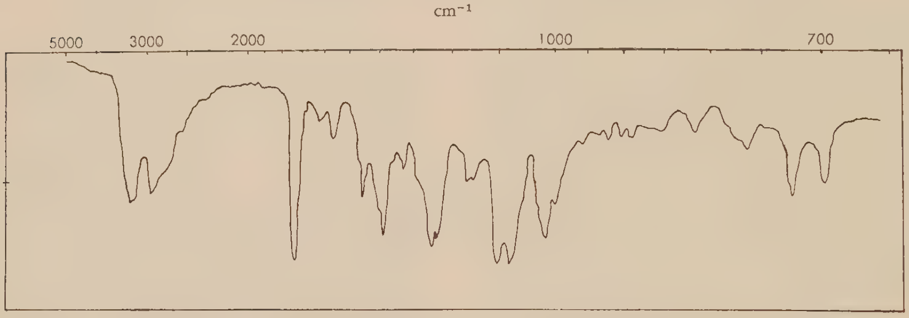


FIG. 1 e. Infrared Spectrum of 1-Deoxy-1-glycino-4,6-benzylidene-D-fructose (KBr).

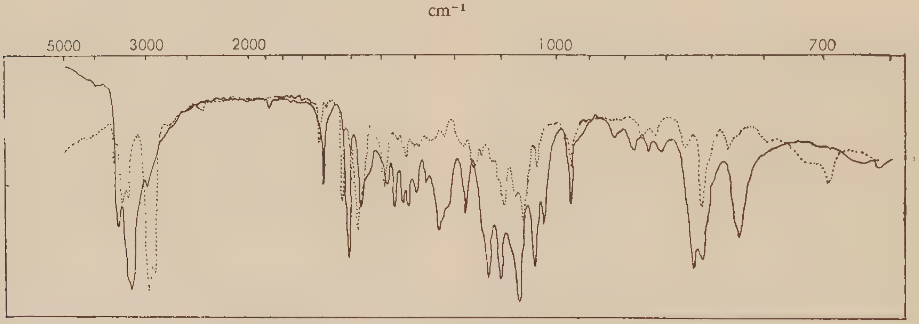


FIG. 1 f. Infrared Spectra of 1-Deoxy-1-*p*-toluidino-D-fructose (—) KBr and N-Benzyl-(1-deoxy-1-*p*-toluidino)-D-fructosylamine (Nujol).

form from the rearrangement medium of glycine-N-D-galactoside (Na salt) as well as from the browning mixture consisting of glycine and D-galactose. The Amadori rearrangement product had the melting point of 165∼168°C (dec.) and R_F of 0.14 (ascending, BuOH-EtOH-water : 40-11-19). The characteristic color reactions for isoglucosamine were also positive with the isogalactosamine.

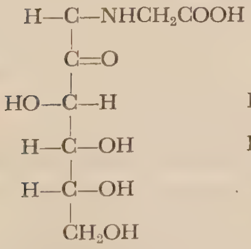
The infrared spectrum (Fig. 1 b) showed a characteristic absorption at 3450 cm^{-1} which is of identical feature with that at 3570 cm^{-1} exhibited by 1-deoxy-1-*p*-toluidino-D-fructose[6] (Fig. 1 f) and by N-benzyl-(1-deoxy-1-*p*-toluidino)-D-fructosylamine (Fig. 1 f) prepared in this laboratory. In contrast, amino acid-N-glycosides

(Fig. 1 a and c) all showed no characteristic absorption near 3500 cm^{-1} and 4,6-benzylidene-1-deoxy-1-glycino (isopropyl ester)-D-fructose[9)] (Fig. 1 e) also did not absorb at 3450 cm^{-1}. It is notable that the infrared spectra of isogalactosamine showed no characteristic absorption band at 3450 cm^{-1}; instead, a broad band near 3300 cm^{-1} was absorbed (Fig. 1 d). It is, therefore, concluded from the results obtained so far with amino acid-isoglucosamine and isogalactosamine that the sharp absorption near 3500 cm^{-1} is exclusively exhibited by isoglucosamine.

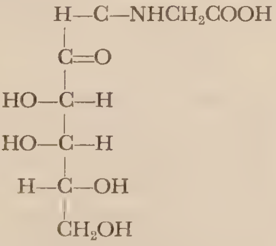
It is questionable to assume that the crystals of glycine-isoglucosamine are identical with the substance obtained by Richards[5)] since he described no melting point, and, in addition, the infrared absorption spectra of both preparations appear to have difference in the major absorption bands. However, it is unlikely that the absoption at 1620 cm^{-1} as interpreted by Richards is due to the presence of the structure –NH–CH=C(OH)–.

The periodate oxidation of glycine-isoglucosamine showed consumption of an amount of 5 moles of periodate which is in agreement with the figure consumed by the substance reported by Richards[6)]. Glycine-isogalactosamine also consumed 5 moles of periodate (Table I). On so far as the oxidation data here obtained, both 1-amino acid-1-deoxy-ketoses appear to have the straight chain structure as shown below. For N-*p*-tolyl-D-isoglucosamine, however, Kuhn and Krüger[17)] have presented the pyranose ring structure.

As has been observed with 1-deoxy-1-*p*-toluidino-D-fructose, amino acid-isoglucosamine is stable and could be kept for some time without undergoing self-browning. It appears that this Amadori rearrangement product is the stabilized product which might undergo destruction when catalyzed by acid or bass as is the case with 1-deoxy-1-*p*-toluidino-D-fructose[12)]. The role in the brown color formation of the Amadori rearrangement and of the destruction of the product is certainly significant. The mechanism of the brown color formation is still mostly obscure and the possible intermediates are yet uncertain, since the browning reaction is a complicated one. Investigations on the intermediates of the reaction are in course.



Glycine-isoglucosamine



Glycine-isogalactosamine

EXPERIMENTAL

Amino acid-N-glycosides. Amino acid-N-glycosides were prepared by the procedures of Weitzel et al.[15)].

Glycine-N-D-glucoside (Na salt). D-Glucose (4 g), glycine Na salt (1.8 g) and methanol (150 ml) were stirred for 2 hours at room temperature. The reaction mixture was filtered to remove insoluble precipitate and to the filtrate were added ethanol and then ether. The formed precipitate was collected by centrifugation and washed twice with ethanol; yield 4.8 g, m.p. 115∼125°C (dec.). *Anal.* Found: N, 5.00; Na_2O, 24.2. Calcd. for $C_8H_{14}NO_7Na$: N. 5.40: Na_2O 23.9.

Glycine-N-D-mannoside (Na salt). D-Mannose (4 g), glycine Na salt (2.16 g) and methanol (300 ml) were stirred at 60°C to a solution containing some insoluble matter. It was filtered and the filtrate was allowed to stand at room temperature for 24 hours during which time crystals separated; yield 3.95 g, m.p. 130∼140°C (dec.). *Anal.* Found: N, 5.27. Calcd. for $C_8H_{14}NO_7Na$: N, 5.40.

Glycine-N-D-galactoside (Na salt). A mixture of D-galactose (4 g), glycine Na salt (2.16 g) and methanol (150 ml) was stirred at 50°C; yield 2.5 g, m.p. 80∼83°C (dec.). *Anal.* Found: N, 5.18. Calcd. for $C_8H_{14}NO_7Na$: N, 5.40.

Glycine-isoglucosamine by the rearrangement of glycin-N-D-glucoside. Glycine-N-D-glucoside (Na salt) (16 g), acetic acid (4.7 ml) and potassium acetate (7.85 g) were refluxed for 4 hours in 1.6 liters of methanol. The reaction mixture was passed through a column of Dowex 50×4 (H^+ form, mesh 100∼200) (2.4×40 cm), which was then eluted with 0.2 N aqueous ammonia. The fractions which showed positive test with ferricyanate

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[17)] R. Kuhn and G. Krüger, *Ann.* **618**, 82 (1958).

were collected and concentrated under reduced pressure. The concentrated solution was passed through a charcoal layer which was then washed with methanol. The combined filtrate and washings were passed through a column of Dowex 50 (H^+ form) (1 cm × 20 cm) which was then eluted with 0.1 N aqueous ammonia. The fractions of the effluent, which showed one spot of R_F 0.16 were collected and concentrated under reduced pressure. The syrup was crystallized three times from water-methanol, m.p. 155°C (dec.), yield 300 mg. *Anal.* Found: C, 40.50; H, 6.38; N, 5.90. Calcd. for $C_8H_{15}NO_7$: C, 40.23; H, 6.28; N, 6.11.

Isolation of glycine-isoglucosamine from the browning reaction mixture of glycine and D-glucose.

(a) D-Glucose (18 g) and glycine (7.5 g) were dissolved in 25 ml of water and the reaction mixture was heated for 1 hour on a water bath. The resultant colored solution was passed through a column (2.5 cm × 25 cm) of Dowex 50 (H^+ form). The column was washed with about 200 ml of water until the effluent showed a negative test with Fehling solution, and was then eluted with 0.1 N aqueous ammonia. The effluent was collected on fraction collector and the fractions which showed one spot of R_F 0.16 were collected (about 100 ml) and concentrated under reduced pressure. The syrup was crystallized from water by gradual addition of methanol, yield 0.6 g, m.p. 156° (dec.), $[\alpha]^{18}_{D}-12°$ (10 min.), −31° (30 min.), −45° (90 min., const.) (*c* 1, water). Borsook reports $[\alpha]^{25}_{D}-65°$ and Anet $[\alpha]^{20}_{D}-68.8°$ (const., 30 min.; *c* 1.2, water). *Anal.* Found: C, 40.47; H, 6.38; N, 5.62. Calcd. for $C_8H_{15}NO_7$: C, 40.50; H, 6.38; N, 5.90.

(b) Glycine (30 g) and D-glucose (72 g) were dissolved in water (80 ml) and the solution was concentrated under reduced pressure to about one forth of the original volume. The solution was allowed to stand overnight at room temperature and was then placed in an incubator at 50°C for 70 hours, during which time the solution became reddish brown. The mass was dissolved in water and the solution was passed through a column of Dowex 50 followed by the same treatment as described above. The colored mass obtained upon evaporation of the effluent was extracted with 85% methanol and the solution was concentrated under reduced pressure. The pale yellow colored syrup was crystallized from water by gradual addition of methanol, m.p. 156°C (dec.), yield 1.1 g.

Glycine-isoglucosamine by the rearrangement of glycine-N-D-mannoside (Na salt). Glycine-N-D-mannoside (Na salt) (16 g) was refluxed for 4 hours in methanol under the conditions described for the rearrangement of glycine-N-D-glucoside. The fractions containing Amadori rearrangement product of the effluent from a Dowed 50×8 (H^+ form) column eluted with 0.2 N aqueous ammonia were concentrated under reduced pressure to a syrup. This was dissolved in aqueous ethanol and the solution was added onto a cellulose powder column (100 ml), which was eluted with 0.1 N aqueous ammonia. The fractions showing one spot on paper chromatogram of the effluent were collected and concentrated under reduced pressure to a syrup. This was crystallized from water and methanol, and recrystallized three times from the same solvent, m.p. 156°C (dec.), yield 360 mg. *Anal.* Found: C, 40.50; H, 6.36; N, 6.13. Calcd. for $C_8H_{15}NO_7$: C, 40.50; H, 6.38; N, 5.90.

The infrared spectrum of this preparation was identical with those of the isoglucosamine prepared from glycine-N-D-glucoside and also from the reaction mixture of glycine and glucose.

1-Deoxy-1-glycino-D-tagatose.

(a) From glycine-N-D-galactoside. Glycine-N-D-galactoside (Na Salt) (16 g), acetic acid (4.7 ml), potassium acetate (7.85 g) and methanol (1.6 liter) were refluxed for 4 hours. The reaction mixture gave a little amount of precipitate which was redissolved by the addition of water. The solution was passed through a column of Dowex 50×8 (H^+ form) (2.5 cm × 20 cm), which was then eluted with 0.2 N aqueous ammonia. The fractions containing the Amadori rearrangement product of the effluent were concentrated under reduced pressure below 25°C to a syrup which was dissolved in 100 ml of methanol. The solution was passed through a charcoal column (100 ml) which had been washed with methanol. The clear effluent was passed through a column (1 cm × 20 cm) of Dowex 50×8 (H^+ form) and the fractions showing one spot on the paper chromatogram of the effluent were collected and concentrated under reduced pressure to a syrup. This was crystallized from water and methanol. Recrystallizations from the same solvent gave a product having m.p. 165∼168°C (dec.), $[\alpha]^{21}_{D}-15°$ (10 min.), −11° (24 hours) (*c* 1.14, water); yield 850 mg. *Anal.* Found: C, 40.60; H, 6.37; N, 5.80. Calcd. for $C_8H_{15}NO_7$; C, 40.50; H, 6.38; N, 5.90.

(b) From the reaction mixture of glycine and galac: tose. D-Galactose (18 g) and glycine (7.9 g) were allowed to react in water (50 ml) at 100°C for 1 hour and the reaction mixture was treated in the procedure as described above. There were obtained crystals melting at 165∼168°C (dec.), in a yield of 100 mg. No depression in m.p. was observed on admixture with the compound from glycine-N-D-galactoside. Infrared spectra of both preparations were also identical. *Anal.* Found: N, 5.94. Calcd. for $C_8H_{15}NO_7$: N, 5.90.

Paper chromatography. The fractions of the effluent were tested paper chromatographically using BuOH-EtOH-water (40-11-19) as the solvent by ascending method on Toyo Roshi No. 51 filter paper. The paper chromatograms were sprayed with (a) aniline hydrogen phthalate, (b) 0.2% ninhydrin in water-saturated butanol or (c) ferricyanate solution. R_F values are shown below.

	R_F
D-Glucose	0.32
Glycine	0.20
1-Deoxy-1-glycino-D-fructose	0.16 ($R_{glucose}$ 0.51 ; $R_{glycine}$ 0.82)
D-Galactose	0.27
1-Doexy-1-glycino-D-tagatose	0.14

N-Benzyl-(1-deoxy-1-*p*-toluidino)-D-fructosylamine. N-*p*-Tolyl-D-isoglucosamine (25 g) was dissolved in benzyl-amine (100 g) and the reaction solution was allowed to stand at room temperature for several days during which time crystals deposited gradually. This was placed in an ice box for another several days and to this was added a mixture of ether and petroleum ether, stirred, filtered and washed with ether and acetone; yield 30 g, m.p. 105~112°C. After recrystallization from ethyl acetate, m.p. 116~118°C, $[\alpha]_D^{22}$ $-62°$ (20 min.), 15° (24 hours) (*c* 1, MeOH). *Anal.* Found: C, 66.75 ; H, 7.19 ; N, 7.55. Calcd. for $C_{20}H_{26}N_2O_4$: C, 67.02 ; H, 7.31 ; N, 7.81.

Infrared spectra. The infrared spectra of N-glycosides and isoglycosamines were recorded by a Perkin-Elmer model infrared spectrophotometer using Nujol mull or pellet (KBr).

Periodate oxidations of 1-deoxy-1-glycino-D-fructose and 1-deoxy-1-glycino-D-tagatose. An amount (23.7

TABLE I. CONSUMPTION OF PERIODATE BY GLYCINE-ISOGLYCOSAMINE

(A) CONSUMPTION BY 1-DEOXY-1-GLYCINO-D-FRUCTOSE

(mole/mole)

Time (min.) pH	15	50	120	250	480	1260	4080	6720
3.2*	3.47	3.57	3.77	3.92	4.42	4.42	4.42	4.57
4.4*	3.82	3.87	4.07	4.22	4.42	4.67	4.52	4.77
5.3*	3.92	3.97	4.07	4.17	4.42	4.57	4.62	4.62
7.1**	4.57	4.67	4.77	4.91	5.17	5.67	5.67	5.67

(B) CONSUMPTION BY 1-DEOXY-1-GLYCINO-D-TAGATOSE

(mole/mole)

Time (min.) pH	15	50	120	240	480	1260	3720	6840
3.2*	2.64	3.19	3.32	3.62	3.97	4.25	4.82	4.98
4.4*	3.04	3.42	3.47	3.75	3.97	4.25	4.53	4.66
5.3*	3.57	3.57	3.67	3.95	4.12	4.48	4.46	4.50
7.1**	3.42	3.50	3.72	3.87	4.08	4.35	4.55	4.70

* Acetate Buffer

** Borate Buffer

TABLE II. FORMIC ACID FORMATION BY GLYCINE-ISOGLYCOSAMINE ON PERIODATE OXIDATION

(A) FORMIC ACID FORMATION BY 1-DEOXY-1-GLYCINO-D-FRUCTOSE (mole/mole)

Time (min.)	15	50	120	240	480	1620	3180	6120
Formic Acid/Sample	1.92	2.65	2.73	2.79	2.73	2.72	2.81	2.86

(B) FORMIC ACID FORMATION BY 1-DEOXY-1-GLYCINO-D-TAGATOSE (mole/mole)

Time (min.)	15	50	120	240	480	1260	3720	6840
Formic Acid/Sample	—	1.66	1.87	1.98	2.18	2.41	2.60	2.70

mg) of the compound was dissolved in 30 ml of buffer solution at 0°C and to this was added 0.2 N sodium metaperiodate solution (10 ml), filled up to 50 ml, and the reaction solution was left in the dark at 0°C. An aliquot (5 ml) was taken at intervals and 0.05 N sodium arsenite solution (5 ml), an excess of sodium hydrogen carbonate and 20% potassium iodide solution (1 ml) were added, and the reaction solution was left for 15 min. at room temperature. This was submitted to back titration with 0.05 N iodine solution using 4 drops of 0.5% starch solution as the indicator. The reaction was done at pH 3.2, 4.4, 5.3 and 7.1, and the results are shown in Table I.

Determination of formic acid in the oxidative reaction solution was carried out as follows. An aliquot (5 ml) of the oxidation reaction solution was taken at intervals, and to this was added ethylene glycol (1 ml). The solution was left in the dark for 10 min. and was then titrated with 0.1 N sodium hydroxide, using methyl red-methyleneblue as the indicator. In this series of experiments for formic acid determination, water was used in place of buffer solution, and the results are given in Table II.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 711～715, 1960]

Studies on Phenolic Lactones

Part IV. Preparation of α-Exo-unsaturated-γ-butyrolactones

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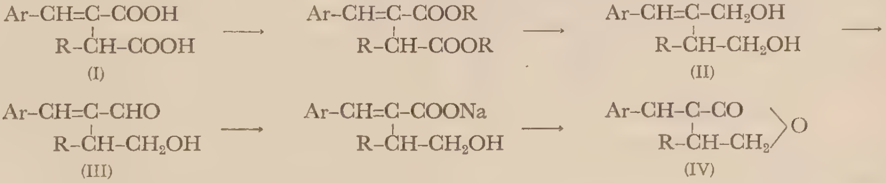
Received June 13, 1960

α-Exo-unsaturated-γ-butyrolactones were prepared. Reduction of α-benzylidene succinic acid esters with lithium aluminum hydride afford 2-benzylidene-1,4-butanediols. Manganese dioxide oxidation of glycols followed by silver oxide oxidation and lactonization afford α-benzylidene-γ-butyrolactones. Thus α-benzylidene-, α-anisylidene-, α-veratrylidene-, α-(3,4,5-trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl)-γ-butyrolactone were synthesized.

The authors were interested in synthesizing certain α-benzylidene-γ-butyrolactone homologues in order to investigate some of their synergistic activity. In the preceding paper[1] the authors reported the synthesis of hibalactone using the manganese dioxide oxidation of unsaturated alcohol. In this paper the preparation of some of α-benzylidene-γ-butyrolactones were described.

For the syntheses of α-substituted-γ-butyrolactones various methods have been reported in the literature. Lactonization of appropriate γ-hydroxy or γ-halo acids, condensation of malonic esters with ethylene oxide afford α-alkyl-γ-butyrolactones. But for the preparation of α-exo-unsaturated-γ-butyrolactones the condensation of aldehydes with butyrolactone is the only method appeared in the literature[2-4].

The authors now adopted the manganese dioxide oxidation method for the syntheses of α-exo-unsaturated butyrolactones as follows:



$$\begin{array}{l} \text{Ar-CH=C-COOH} \\ \quad\;\; \text{R-CH-COOH} \\ \quad\;\; \text{(I)} \end{array} \longrightarrow \begin{array}{l} \text{Ar-CH=C-COOR} \\ \quad\;\; \text{R-CH-COOR} \end{array} \longrightarrow \begin{array}{l} \text{Ar-CH=C-CH}_2\text{OH} \\ \quad\;\; \text{R-CH-CH}_2\text{OH} \\ \quad\;\; \text{(II)} \end{array} \longrightarrow$$

$$\begin{array}{l} \text{Ar-CH=C-CHO} \\ \quad\;\; \text{R-CH-CH}_2\text{OH} \\ \quad\;\; \text{(III)} \end{array} \longrightarrow \begin{array}{l} \text{Ar-CH=C-COONa} \\ \quad\;\; \text{R-CH-CH}_2\text{OH} \end{array} \longrightarrow \begin{array}{l} \text{Ar-CH-C-CO} \\ \quad\;\; \text{R-CH-CH}_2 \\ \quad\;\; \text{(IV)} \end{array} \!\!\Big\rangle \text{O}$$

(Ar = C_6H_5, p-CH_3O-C_6H_4, 3,4-$(CH_3O)_2C_6H_3$, 3,4,5-$(CH_3O)_3C_6H_2$, R = H or Ar)

In the first step the authors attempted the oxidation of 2-benzylidene-1,4-butanediols. Although, oxidation of allyl type alcohol to α, β-unsaturated aldehyde using manganese dioxide powder in organic solvent have many uses in steroid and polyene alcohol fields, but in the case of alcohol having only one double bond oxidation does not proceed smoothly as usual. As a matter of fact oxidation of some of the 2-benzylidene-1,4-butanediols (II) to corresponding hydroxy aldehyde (III) proceeded only in low yield and hence the yield of α-benzylidene-γ-butyrolactone were rather poor.

The authors were not interested in elaboration of the optimal conditions in every case. With any glycol, only one or two experiments were run, thus the yields given may not represent the maximal ones obtainable. But for

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1) K. Yamashita and M. Matsui, This Bulletin, **24**, 160 (1960).
2) K. Yamashita and M. Matsui, *ibid.*, **23**, 230 (1959).
3) A. R. Pinder, *J. Chem. Soc.*, **1952**, 2236.
4) H. Zimmer and J. Rothe, *J. Org. Chem.*, **24**, 28 (1959).

the synthesis of a series of α-benzylidene-γ-butyrolactones the condensation of aldehydes with butyrolactone seems superior method than that described here. For the preparation of α-benzylidene-β-benzyl-γ-butyrolactone homologue manganese dioxide oxidation may be valuable method as shown in the synthesis of hibalactone, and so we used this method to prepare α-exo-unsaturated butyrolactones.

To synthesize α-benzylidene-β-benzyl-γ-butyrolactones α-benzylidene-β-benzyl succinic acid homologues must be preparad as the intermediate. The example of the Stobb condensation of aryl aldehyde with alkyl benzylsuccinate were unexpectedly few in the literature. Up to date only few preparations were reported in the literature, that is: α-benzylidene-β-benzyl-[5], α-anisylidene-β-benzyl-[6], α-3,4,5-trimethoxybenzylidene-β-piperonyl-[7] succinic acid. The authors attempted the synthesis of α-anisylidene-β-anisyl succinic acid and α-(3,4,5-trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl) succinic acid. The Stobbe condensation proceeded normally when sodium ethylate in alcohol was used as condensing agent, and desired benzyl benzylidene succinic acids were obtained. These substituted acids were esterified and reduced with lithium aluminum hydride to glycols. The oxidation of glycols with manganese dioxide did not proceed smoothly, but after silver oxide oxidation and lactonization a small amounts of oily lactone fractions were obtained. After standing several weeks in refrigerator α-trimethoxybenzylidene-β-trimethoxybenzyl-γ-butyrolactone was obtained as two kinds of crystalline needles probably cis and trans isomer, but α-anisylidene-β-anisyl-γ-butyrolactone could not be crystallized.

EXPERIMENTAL

The following compounds were prepared according to standard procedures. Benzylidene succinic acid[8], m.p. 185～187°C; diethyl ester[9], b.p. 180～185°C/10 mm; anisylidene succinic acid[10], m.p. 188～190°C; dimethyl ester[11], b.p. 165°C/0.5 mm; veratrylidene succinic acid[10], m.p. 173～175°C; piperonylidene succinic acid[10], m.p. 190～194°C.

α-Benzylidene-γ-butyrolactone (General procedure) A solution of 10 g of diethyl benzylidene succinate in 100 ml of dry ether was added dropwise to a suspension of 1.4 g of lithium aluminum hydride in 50 ml of anhydrous ether at a rate so as to produce gentle reflux. After refluxing for an hour, the reaction mixture was destroyed by addition of ethyl acetate, water and 40 ml of 15% hydrochloric acid. The ether layer was separated and the solvent was removed by distillation under atmospheric pressure. The residual oil was refluxed with 50 ml of 5% methanolic potash for an hour to destroy any unreacted ester. After the addition of 50 ml of water, methanol was distilled off and glycol was extracted with ether. Evaporation of ether left 8.0 g of oily 2-benzylidene-1,4-butanediol.

To a solution of glycol, 8.0 g, in 80 ml of acetone precipitated manganese dioxide powder (25 g) was added and left standing for 20 hours with occasional shaking. Manganese dioxide was filtered and washed with acetone, and the combined filtrate was concentrated to syrup. The oily residue containing α-benzylidene-γ-hydroxybutyraldehyde was obtained, weighed 7.5 g.

The oily residue (7.5 g) was dissolved in 20 ml of warm ethanol and 100 ml of 5% sodium hydroxide solution was added. Silver oxide freshly prepared from 20 g of silver nitrate was added and warmed at 50～60°C for an hour with vigorous stirring. The hot mixture was filtered and ethanol was distilled off. After the removal of neutral substances the mother liquor was acidified with hydrochloric acid and warmed on water bath for 30 minutes. The reaction mixture was carefully poured into excess of sodium bicarbonate solution and extracted three times with chloroform. The combined extract was washed with water and dried over sodium sulfate. After evaporation of chloroform 1.8 g of crude lactone fraction was obtained. After twice recrystallization from methanol α-benzylidene-γ-butyrolactone was obtained as white needles, m.p. 115～117°C. *Anal.* Found: C, 75.60; H, 5.49. Calcd. for $C_{11}H_{10}O_2$: C, 76.0; H, 5.6.% Infra-red spectrum showed strong absorption at 1746 cm^{-1} corresponding to α,β-unsaturated

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[6] Cordier, *Ann. Chim.*, (10) **15**, 228 (1931).

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[11] A. L. J. Buckle, A, Mc. Gookin and A. Robertson, *J. Chem. Soc.*, **1954**, 3981.

γ-lactone.

α-Anisylidene-γ-butyrolactone

From 6 g of diethyl anisylidene succinate 0.42 g of α-anisylidene-γ-butyrolactone was obtained as white needles, m.p. 125~127°C. *Anal.* Found: C, 71.12; H, 6.16. Calcd. for $C_{12}H_{12}O_3$: C, 70.6; H, 5.8%. Infra-red spectrum showed strong absorption at 1740 cm^{-1}.

α-Veratrylidene-γ-butyrolactone

From 15 g of dimethyl veratrylidene succinate 0.7 g of α-veratrylidene-γ-butyrolactone was obtained as a needle, m.p. 115~117°C. *Anal.* Found: C, 66.95; H, 6.10. Calcd. for $C_{13}H_{14}O_4$: C, 66.7; H, 6.0%.

α-Piperonylidene-γ-butyrolactone

From 15 g of dimethyl piperonylidene succinate, m.p. 78~79°C, 0.3 g of α-piperonylidene-γ-butyrolactone was obtained as needles, m.p. 177~178°C. *Anal.* Found: C, 66.16; H, 4.95. Calcd. for $C_{12}H_{10}O_4$: C, 66.1; H, 4.6%. Infra-red spectrum showed absorption at 1740 cm^{-1}.

α-(3, 4, 5-Trimethoxybenzylidene)-β-(3, 4, 5-trimethoxybenzyl)-γ-butyrolactone.

a) Dimethyl 3,4,5-trimethoxybenzyl succinate:

Lithium aluminum hydride reduction of 50 g of methyl 3,4,5-trimethoxybenzoate afforded 38.5 g of 3,4,5-trimethoxybenzyl alcohol, b.p. 165~166°C/7 mm.

3,4,5-Trimethoxybenzyl alcohol, 38.5 g, was dissolved in 300 ml of benzene and 24 g of sodium sulfate was added. While the mixture was being cooled at 0~5°C, dry hydrogen chloride was passed for 30 minutes and left standing for several hours in the cold. The filtered solution was washed well with water. Evaporation of the solvent left 43.8 g of trimethoxybenzyl chloride which gradually solidified, m.p. 59~60°C. After recrystallization from benzene-petroleum ether the pure chloride melted at 61°C. *Anal.* Found: C, 55.84; H, 6.10. Calcd. for $C_{10}H_{13}O_3Cl$: C, 55.42; H, 6.05%.

Metalic sodium (3.1 g) was dissolved in 100 ml of absolute ethanol and 33 g of diethyl carbethoxymethyl malonate was added. Then 28 g of 3,4,5-trimethoxybenzyl chloride in 50 ml of ethyl alcohol was added dropwise. The mixture was heated under reflux for 3 hours. A solution of 30 g of potassium hydroxide in 100 ml of ethyl alcohol and 20 ml of water was added dropwise and refluxed for an hour. One hundred milliliters of water was added and the alcohol was distilled off. After cooling the trimethoxybenzyl succinic acid was extracted with ethyl acetate. Evaporation of

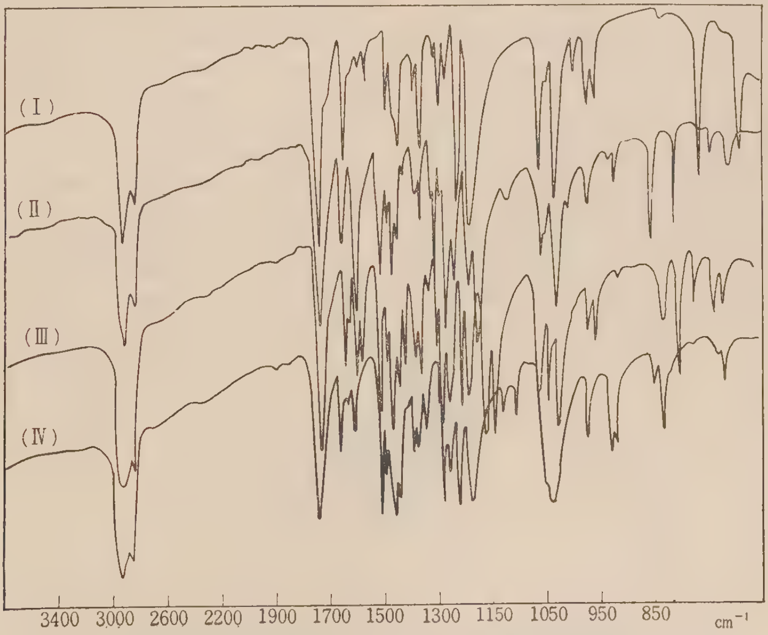

FIG. 1. Infrared Absorption Spectra of α-Benzylidene-γ-butyrolactone.

(I) α-Benzylidene-γ-butyrolactone (II) α-Anisylidene-γ-butyrolactone
(III) α-Veratrylidene-γ-butyrolactone (IV) α-Piperonylidene-γ-butyrolactone

the solvent left 35 g of crude acid. Esterification of crude acid with methanol-sulfuric acid and distillation of the ester fraction afforded 18.3 g of dimethyl 3,4,5-trimethoxybenzyl succinate, b.p. 194~196°C/4 mm. The distillate solidified gradually, m.p. 59°C. *Anal.* Found: C, 58.84; H, 6.7. Calcd. for $C_{16}H_{22}O_9$: C, 58.9; H, 6.7%. Hydrolysis of the ester afforded 3,4,5-trimethoxybenzyl succinic acid, m.p. 151°C.

b) α-(3,4,5-Trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl) succinic acid:

To a solution of 2.8 g of sodium in 50 ml of anhydrous ethanol was added a solution of 11.0 g of 3,4,5-trimethoxybenzaldehyde and 18.3 g of dimethyl 3,4,5-trimethoxybenzyl succinate in 50 ml of ethanol and the mixture was heated under reflux for 3 hours. After the addition of 50 ml of water the alcohol was removed by distillation. Neutral substances were removed by ether extraction and the aqueous solution was acidified with hydrochloric acid. Extraction with ethyl acetate and evaporation of solvent left 24.7 g of oily residues upon which treatment with 50 ml of ether gradually separated 9.8 g of trimethoxybenzylidene- trimethoxybenzyl succinic acid as white powder, m.p. 160~161°C. *Anal.* Found: C, 58.21; H, 6.06. Calcd. for $C_{24}H_{28}O_{10}H_2O$: C, 58.4; H, 6.1%.

From the analytical value the product seemed hydrate or hydroxy acid. But as the esterification of this acid with diazomethane afforded unsaturated ester the acid obtained above seemed to be α-(3,4,5-trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl) succinic acid hydrate.

c) α-(3,4,5-Trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl) succinic acid dimethyl ester:

The substituted succinic acid, 7.1 g, was esterified with ethereal solution of diazomethane prepared from 5.5 g of nitrosomethyl urea. After the evaporation of ether residual ester was recrystallized from methanol, m.p. 127~128°C. *Anal.* Found: C, 61.80; H, 6.23. Calcd. for $C_{26}H_{32}O_{10}$: C, 61.90; H, 6.3%.

d) α-(3,4,5-Trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl)-γ-butyrolactone:

To a solution of 1.1 g of lithium aluminum hydride in 100 ml of ether was added dropwise a solution of dimethyl ester in 50 ml of tetrahydrofuran. After refluxing for one hour the reaction mixture was decomposed with ethyl acetate and a small quantity of water. The organic layer was decanted from slurry mass. After evaporation of the solvent, residual mass was saponified with methanolic potash. After the removal of alcohol the glycol was extracted with ether. Evaporation of ether left 8.1 g of oily 2-(3,4,5-trimethoxybenzylidene)-3-(3,4,5-trimethoxybenzyl)-butanediol (1,4).

To a solution of glycol in 80 ml of acetone was added 35 g of precipitated manganese dioxide powder and left standing for 24 hours with occasional shaking. The filtrate was concentrated to syrup. The residual crude hydroxy aldehyde was dissolved in 20 ml of warm ethanol and was added to a stirring suspension of 4 g of silver oxide in 40 ml of 5% aqueous sodium hydroxide solution and warmed for an hour at 50~60°C with vigorous stirring. After the removal of ethanol from the filtered solution, the neutral substances were removed by ether extraction. The alkaline mother liquor was acidified with hydrochloric acid and warmed on water bath for 30 minutes to complete lactonization. The lactone was extracted with chloroform and washed with aqueous sodium bicarbonate solution and water. After evaporation of the solvent 1.5 g of oily lactone fraction was obtained. When the methanolic solution of lactone fraction was set aside several weeks in refrigerator some crystals were separated. After twice recrystallization from methanol the white needles were obtained, m.p. 90~91°C. *Anal.* Found: C, 64.07; H, 6.57. Calcd.

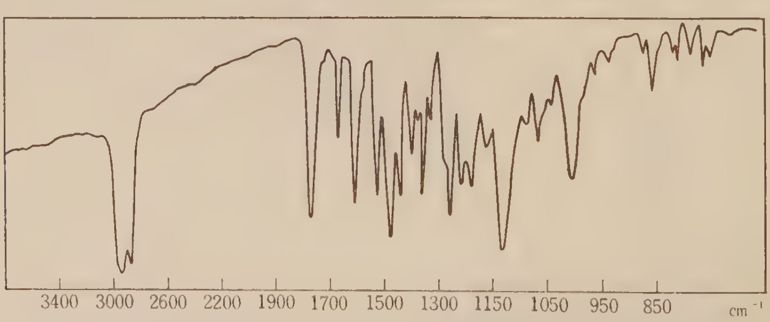

FIG. 2. Infrared Absorption Spectra of α-(3,4,5-Trimethoxybenzylidene.) β-(3,4,5-trimethoxybenzyl)-γ-butyrolactone (Nujol mull)

for $C_{24}H_{28}O_8$ requires: C, 64.8; H, 6.3%. Infra-red spectrum showed strong absorption at 1763 cm^{-1}. (Fig. 2)

From the filtrate of first crystals another crystals were precipitated upon standing. After twice recrystallization it melted at 125～126°C. *Anal.* Found: C, 64.47; H, 6.17. Calcd. for $C_{24}H_{28}O_8$: C, 64.8; H, 6.3%. These two crystals seemed to be *cis* and *trans* isomers of α-(3,4,5-trimethoxybenzylidene)-β-(3,4,5-trimethoxybenzyl)-γ-butyrolactone.

Attempted synthesis of α-Anisylidene-β-anisyl-γ-butyrolactone

a) Dimethyl anisylsuccinate:

Anisyl chloride was condensed with diethyl carbethoxy methyl malonate as described before. Saponification, decarboxylation and esterification afforded dimethyl anisyl succinate, b.p. 157～160°C/4 mm, n_D^{16}1.5360. *Anal.* Found: C, 63.54; H, 6.41. Calcd. for $C_{14}H_{18}O_5$: C, 63.1; H, 6.8%.

Hydrolysis of the ester yielded anisyl succinic acid, m.p. 101°C.

b) α-Anisylidene-β-anisyl succinic acid:

Anisaldehyde was condensed with dimethyl anisyl succinate in the presence of sodium ethylate in ethyl alcohol. The acid fraction gradually solidified. After recrystallization from ethyl acetate-petroleum benzine α-anisylidene-β-anisyl succinic acid was obtained as white needles, m.p. 227～228°C. *Anal.* Found: C, 67.4; H, 4.93. Calcd. for $C_{20}H_{20}O_6$: C, 67.4; H, 5.6%.

c) Attempted synthesis of α-anisylidene-β-anisyl-γ-butyrolactone:

α-Anisylidene-β-anisyl succinic acid was esterified with diazomethane. The oily ester was reduced with lithium aluminum hydride to glycol. Manganese dioxide oxidation of glycol in acetone followed by silver oxide oxidation and lactonization afforded a small amount of lactone fraction, which could not be crystallized.

Acknowledgements The authors express their thanks to the members of the Analytical Laboratory of the Department of Agricultural Chemistry, University of Tokyo, for microanalysis. The infrared spectrum are due to K. Aizawa, to whom the authors are grateful.

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Studies on Phenolic Lactones

Part V. General Route to Substituted β-Benzyl-γ-butyrolactones and an Alternate Synthesis of (±) Hibalactone

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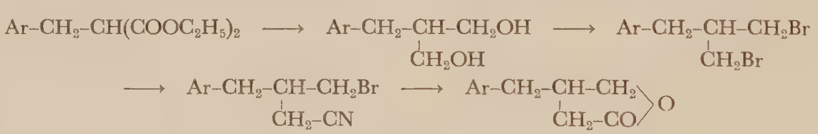
Received June 13, 1960

Substituted β-benzyl-γ-butyrolactones were synthesized. Hydrolysis and decarboxylation of ethyl α-allyl-α-benzyl malonate gave α-allyl-α-benzyl acetic acid. Esterification of the acid and subsequent reduction with lithium aluminum hydride afforded β-benzyl-β-allyl ethanol. Oxidation of the double bonds with potassium permanganate followed by periodate treatment, silver oxide oxidation and lactonization yielded β-benzyl butyrolactones. Thus β-benzyl-, β-piperonly-, β-3,4,5-trimethoxybenzyl- butyrolactone were prepared.

Condensation of piperonal and β-piperonyl-γ-butyrolactone with sodium ethylate afforded (±) hibalactone.

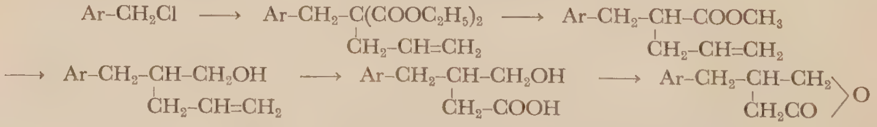
The authors were interested in preparing substituted β-benzyl-γ-butyrolactones as intermediates for further synthetic work. It is a well known fact that the condensation of substituted malonic esters with ethylene oxide or lactonization of γ-haloesters afford α-benzyl-γ-butyrolactones. For the synthesis of substituted β-benzyl-γ-butyrolactones, however, only a few reports have appeared in the literature. Reduction of β-substituted succinic acid β-mono thioalkyl ester with Raney nickel catalyst[1] or hydroxymethylation of β-benzoyl propionic acid followed by catalytic hydrogenation[2] afford substituted β-benzyl butyrolactones.

The authors attempted several other routes for the synthesis of β-benzyl-γ-butyrolactones. For the first time the authors attempted the lactonization of γ-bromo butyronitriles as follows:



$$\mathrm{Ar{-}CH_2{-}CH(COOC_2H_5)_2} \longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2OH}{CH}{-}CH_2OH} \longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2Br}{CH}{-}CH_2Br}$$
$$\longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2{-}CN}{CH}{-}CH_2Br} \longrightarrow \left.\begin{array}{l}\mathrm{Ar{-}CH_2{-}CH{-}CH_2}\\ \qquad\quad \mathrm{CH_2{-}CO}\end{array}\right\rangle \mathrm{O}$$

But in the course of this route the reaction of one mole of sodium cyanide with one mole of dibromide afforded only a small amount of bromo-nitrile and the product was mainly dinitrile and recovered dibromide. Accordingly the yield of β-benzyl butyrolactone was very poor. So the authors devised an another scheme as follows:



$$\mathrm{Ar{-}CH_2Cl} \longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2{-}CH{=}CH_2}{C}(COOC_2H_5)_2} \longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2{-}CH{=}CH_2}{CH}{-}COOCH_3}$$
$$\longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2{-}CH{=}CH_2}{CH}{-}CH_2OH} \longrightarrow \mathrm{Ar{-}CH_2{-}\underset{\displaystyle CH_2{-}COOH}{CH}{-}CH_2OH} \longrightarrow \left.\begin{array}{l}\mathrm{Ar{-}CH_2{-}CH{-}CH_2}\\ \qquad\quad \mathrm{CH_2CO}\end{array}\right\rangle \mathrm{O}$$

Hydrolysis and decarboxylation of ethyl α-allyl-α-benzyl malonate gave α-allyl-α-benzyl acetic acid. Esterification of the acid and subsequent reduction with lithium aluminum hydride afforded β-benzyl-β-allyl ethanol. Oxidation of the double bonds with potassium permanganate followed by periodate treatment, silver oxide oxidation and lactonization yielded β-benzyl butyrolactones. Of course, the ozonization of β-allyl-β-benzyl-ethanol was expected to yield the same results.

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[1] S. Okumura, M. Masumura, T. Horie and S. Kori, *J. Soc. Org. Synth. Chem. Japan*, **17**, 461 (1951).

[2] J. Rothe and H. Zimmer, *J. Org. Chem.* **24**, 586 (1959).

For the synthesis of some of the substituted β-benzyl-γ-butyrolactone reduction of β-benzoyl-γ-butyrolactones seemed to be superior method, however, β-piperonyl butyrolactone was more easily obtained by our method than that from β-piperonoyl butyrolactone.

The authors attempted the condensation of piperonal with β-piperonyl-γ-butyrolactone. Although the condensation of piperonal with β-piperonyl-butyrolactone by the use of potassium amide in liquid ammonia afforded hydroxylactone, the use of sodium ethylate or sodium hydride in benzene gave racemic hibalactone.

EXPERIMENTAL

β-Benzyl-γ-butyrolactone (General procedure)

a) Ethyl α-allyl-α-benzyl acetate

Ethyl allyl malonate (50 g, 0.25 mole) was alkylated with 35 g (0.28 mole) of benzyl chloride in the presence of 5.8 g (0.25 mole) of sodium in absolute ethanol. After refluxing for 2 hr. 30 g (0.54 mole) of potassium hydroxide in 100 ml of water was added and refluxed for an hour to saponify the esters. After the distillation of alcohol neutral substances were removed by ether extraction. An aqueous solution was acidified with hydrochloric acid and extracted with ether. After the evaporation of the ether the residual mass was heated to 170~180°C to decarboxylation. The crude acid was esterified by refluxing with ethanol and sulfuric acid for 2 hr. The neutral fraction was separated and distilled in vacuo. Ethyl α-allyl-α-benzyl acetate was distilled at 120~125°C, yielded 26.2 g. *Anal*. Found: C, 77.32; H, 8.06. Calcd. for $C_{14}H_{18}O_2$: C, 77.1; H, 8.2%.

b) β-Allyl-β-benzyl ethanol

Ethyl α-allyl-α-benzyl acetate, 26.2 g (0.12 mole), was reduced with 2.66 g (0.07 mole) of lithium aluminum

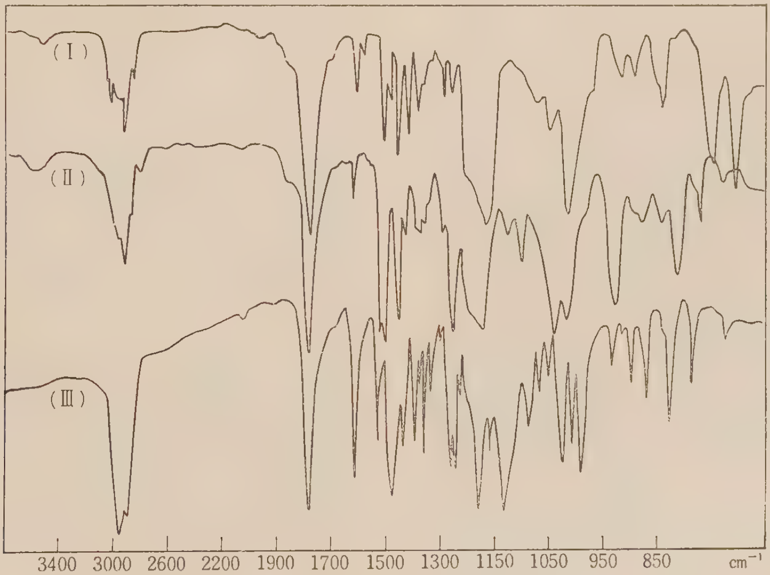

FIG. 1. Infrared Absorption Spectra of β-Benzyl-γ-butyrolactones.

(I) β-Benzyl-γ-butyrolactone (film)
(II) β-Piperonyl-γ-butyrolactone (film)
(III) β-3,4,5-Trimethoxybenzyl-γ-butyrolactone (Nujol mull)

hydride. β-Allyl-β-benzyl ethanol was distilled at 120∼121°C/6 mm, yielded 19.3 g. *Anal.* Found: C, 82.20; H, 9.06. Calcd. for $C_{12}H_{16}O$: C, 81.8; H, 9.1%.

c) **β-Benzyl-γ-butyrolactone**

A solution of 17.4 g (0.11 mole) of potassium permanganate in 350 ml of water was added to a cold solution of 19.3 g (0.11 mole) of β-allyl-β-benzyl ethanol in 100 ml of acetone at 0∼5°C. After stirring for 2 hr. below 10°C and then 1 hour at 60°C, the reaction mixture was filtered to remove manganese dioxide. After the evaporation of acetone from the filtrate, 200 g of potassium carbonate was added. Crude triol was separated as a oily layer, weighted 16.9 g.

Crude 2-benzyl pentanetriol (1,4,5) was dissolved in 50 ml of water and was added to a solution of 18.3 g (0.08 mole) of periodic acid in 100 ml of water with stirring under cooling.

After stirring for an hour, the separated hydroxyaldehyde was extracted with ethyl acetate. Evaporation of the solvent from the extract left 13.8 . of crude β-benzyl-γ-hydroxybutyraldehyde. The crude aldehyde in 50 ml of warm ethanol was added to a suspension of silver oxide freshly prepared from 26.4 g of silver nitrate in 100 ml of 5% sodium hydroxide solution and warmed for 30 minutes at 80°C with vigorous stirring. The hot mixture was filtered and ethanol was distilled off. After the removal of neutral substances with chloroform extraction the aqueous solution was acidified with hydrochloric acid and warmed on water bath for 30 minutes. The reaction mixture was extracted with chloroform, and the extract was washed with sodium bicarbonate solution and water. After removal of solvent, the residual mass was distilled in vacuo. β-Benzyl-γ-butyrolactone was distilled at 165∼166°C/6 mm, weighed 6.4 g. *Anal.* Found: C, 74.07; H, 6.93; Calcd. for $C_{11}H_{12}O_2$: C, 75.0; H, 6.8%. Infra-red spectrum showed a characteristic lactone band at 1740 cm^{-1}.

β-Piperonyl-γ-butyrolactone

Methyl α-allyl-α-piperonyl acetate was prepared from 50 g of allyl malonate and 48 g of piperonyl chloride in the similar manner as described in general procedure. It was distilled at 188°C/21 mm, yielded 32 g. *Anal.* Found: C, 67.81; H, 7.18. Calcd. for $C_{14}H_{16}O_4$: C, 67.8; H, 6.4%.

β-Allyl-β-piperonyl ethanol, b.p. 190°C/14 mm, was obtained by lithium aluminum hydride reduction of the ester in 95% yield. *Anal.* Found: C, 70.75; H, 7.84. Calcd. for $C_{13}H_{16}O_3$: C, 70.9: H, 7.3%.

β-Piperonly-γ-butyrolactone was obtained by the general procedure, b.p. 174∼175°C/0.4 mm, weighed 7.4 g. *Anal.* Found: C, 64.91, H, 5.27. Calcd. for $C_{12}H_{12}O_4$: C, 65.5; H, 5.4%.

β-3,4,5-Trimethoxybenzyl-γ-butyrolactone

Methyl α-allyl-α-(3,4,5-trimethoxybenzyl)-acetate was prepared by the general procedure, b.p. 180∼183°C/6 mm.

β-Allyl-β-(3,4,5-trimethoxybenzyl)-ethanol, b.p. 188∼189°C/6 mm, was prepared by the reduction of the ester in 92% yield. *Anal.* Found: C, 67.48; H, 9.06. Calcd. for $C_{15}H_{22}O_4$: C, 67.7; H, 8.3%.

β-(3,4,5-Trimethoxybenzyl)-γ-butyrolactone was obtained by general procedure, b.p. 168∼170°C/0.1 mm, m.p. 81∼83°C (from methanol). *Anal.* Found: C, 63.04; H, 7.54. Calcd. $C_{14}H_{18}O_5$: C, 63.1; H, 6.8%.

(±) Hibalactone

To a suspension of sodium ethylate prepared from 0.54 g of sodium in dry benzene, a solution of 4.0 g of β-piperonyl-γ-butyrolactone and 3.0 g of piperonal in benzene was added with external cooling. After stirring for 5 hr. at 0∼5°C the reaction mixture was allowed to stand over night. After refluxing for 4 hr. 50 ml of water was added and refluxed for an hour. After cooling the organic layer was discarded and the aqueous solution was acidified with hydrochloric acid and warmed on water bath for 30 minutes. The mixture was extracted with ethyl acetate and the extract was washed with dilute sodium bicarbonate solution and with water, and the ethyl acetate distilled off. The residue was recrystallized from methanol. The colorless plate, m.p. 153∼155°C, was obtained, weighed 1.0 g. Upon admixture with authentic (±) hibalactone the m.p. did not depressed. When sodium hydride was used instead of sodium ethylate, the same results were obtained.

Acknowledgements The authors are grateful to the members of the Analytical Laboratory of the Department of Agricultural Chemistry, University of Tokyo, for microanalytical data. They are also indebted to Mr. K. Aizawa for the measurement of infra-red spectrum.

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Chemistry of the N′-Oxides of Nicotine and Myosmine[1]

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Nicotine-N′-oxide (II) was purified to give a crystalline form which has m.p. 170~171°C, and $[\alpha]_{D}^{21}=+65.3$°C. The reaction of nicotine-N′-oxide with acetic anhydride afforded a good yield of 1′-(3-pyridyl)-4′-(N′-acetylmethylamino)-1′-propanone (V) which was hydrolyzed to give pseudoöxynicotine (III). Nicotine-N′-oxide (II) either with acetyl chloride or with benzoyl chloride, under similar conditions, furnished pseudoöxynicotine (III) without giving the corresponding acyl compound as an intermediate. 2′-Methyl-6′-(3-pyridyl)-tetrahydro-1′,2′-oxazine (XII) rearranged from nicotine-N′-oxide reacted neither with acetic anhydride nor acetyl chloride. Reduction of the oxime (IV) of pseudoöxynicotine dihydrochloride gave dl-1′-amino-1′-(3-pyridyl)-4′-methylaminobutane (VII) as a main product. The hydrazone (VIII) of pseudoöxynicotine dihydrochloride subjected to a modification of the Wolf-Kischner reaction, was reduced to yield dihydrometanicotine (IX). The pyrolysis of N′-methylmyosmine (IV) gave N′-methylnicotinamide (XI) and nicotyrine (X) in low yields. The presence of N′-methylmyosmine in the autoxidation mixture of nicotine was also established. Oxidation of nornicotine (XIV) with hydrogen peroxide furnished myosmine-N′-oxide (XV) whose identity was established by its chemical and physical properties. This oxide, on pyrolysis, gave nornicotyrine (XVII) and myosmine (XVI).

In a continuing study of the chemistry of nicotine degradation, we investigated the behavior of nicotine-N′-oxide (II) and N′-methyl myosmine (IV), because of their potential value as intermediates of nicotine transformation in tobacco leaves. A previous paper[3] from this laboratory has established that N′-methylmyosmine is one of the first intermediates in the degradation of nicotine by soil bacteria. On the basis of their extensive studies[4~7] on the conversion of the tobacco alkaloids in cigar leaves, Frankenburg and his co-workers presented a scheme[6] for a nicotine degradation pathway in which N′-methylmyosmine is postulated as a direct intermediate of nicotine. However, the presence of N′-methylmyosmine, either in tobacco leaves[4~7] or in the oxidation products of nicotine[8~10], has not been proved yet, presumably because of the unstableness of the base, which forms a very viscous and dark brown oil even under refrigeration[11].

On the other hand, nicotine-N′-oxide, is known to be present in tobacco leaves[5] and in the oxidation products of nicotine[8~10] but whether or not it undergoes further transformation has not been proved.

In order to elucidate these points the present work was undertaken and we found that

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[1] A preliminary note of part of this study was published in This Bulletin, **23**, 454 (1659).

[2] His former name was Einosuke Wada.

[3] E. Wada and K. Yamasaki, *J. Am. Chem. Soc.*, **76**, 155 (1954).

[4] W. G. Frankenburg et al., *ibid. Soc.*, **74**, 4309 (1952).

[5] W. G. Frankenburg and A. M. Gottscho, *ibid.*, **77**, 5728 (1955).

[6] W. G. Frankenburg et al., *ibid.*, **77**, 5730 (1955).

[7] W. G. Frankenburg and A. A. Vaitekunas, *ibid.*, **79**, 149 (1957).

[8] C. H. Rayburn, W. R. Harlan and H. R. Hanmer, *ibid.* **63**, 115 (1941).

[9] L. Weil and J. Maher, *Arch. Biochem.*, **29**, 241 (1951).

[10] E. Wada, T. Kisaki and K. Saito; *Arch. Biochem., Biophys.*, **79**, 124 (1959)

[11] P. G. Haines and A. Eisner, *J. Am. Chem. Soc.*, **72**, 1719 (1950).

TABLE I. CONVERSION OF NICOTINE-N′-OXIDE TO PSEUDOÖXYNICOTINE

Method	Nicotine-N′-oxide (g)	Reagent (ml)	Total steam dist. (l)	Practical yield (g)	Theoretical yield (g)	Yield (%)
Pinner and Wolfenstein[12]	26.5	conc. HCl (in a sealed tube) 25	6	10.5	34.8	30
Method A 1	30.5	acetic anhydride 50	9	28.9	42.9	71
2	50.0	acetic anhydride 65	7	41.8	70.4	59
Method B 1	30.5	acetyl chloride 40	5	20.3	43.0	48
2	14.0	acetyl chloride 25	6.5	11.5	19.7	58
Method C	30.0	benzoyl chloride 40	6	22.4	42.3	53

pseudoöxynicotine and thus N′-methylmyosmine which in solution is in equilibrium with pseudoöxynicotine[11] can be isolated from the autooxidation mixture of nicotine and that nicotine-N′-oxide can be rearranged to give N′-methylmyosmine under rather milder conditions.

By exactly following the same procedure as used by Pinner and Wolfenstein[12], Haines and Eisner[11] proved that pseudoöxynicotine, first described by the former workers, is an open chain hydrolytic product of N′-methylmyosmine. In their experiment, the latter workers heated nicotine-N′-oxide in a sealed tube with hydrochloric acid at 140°C for several hours and obtained the dihydrochloride of pseudoöxynicotine in 12% yield. In our present work, nicotine-N′-oxide was treated with acetic anhydride under ice-bath cooling. The reaction mixture was extracted with chloroform after alkalinization and the chloroform extract yielded 1′-(3-pyridyl)-4′-(N′-acetylmethylamino)-1′-propanone (V) upon evaporation and fractional distillation. Hydrolysis of the compound, V, with an alkaline solution gave 60～70% yields of pseudoöxynicotine as dihydrochloride. On treatment either with acetyl chloride or benzoyl chloride, nicotine-N′-oxide yielded, upon alkalinization and extraction with chloroform, N′-methylmyosmine and a trace of the corresponding acyl derivatives. The acyl derivatives should be formed but they may be hydrolyzed by the hydrochloric acid formed during the reaction. So, after alkalinization both of the reaction mixtures were directly steam distilled to yield the dihydrochloride of pseudoöxynicotine. The yields of dihydrochloride of pseudoöxynicotine by the procedures studied in this work are summarizedin Table I.

Rayburn, Harlan and Hanmer[13] obtained a small amount of pseudoöxynicotine upon treating 2′-methyl-6′-(3-pyridyl)-tetrahydro-1′,2′-oxazine (XII), which was derived from nicotine-N′-oxide by rearrangement with hydrochloric acid at 140°C in a sealed tube under the same conditions as employed in the preparation of pseudoöxynicotine. We also applied acetic anhydride and acetyl chloride to the oxazine, but obtained no derivatives. No reaction occurred upon heating the oxazine with acetic anhydride at 70°C for several hours, while on the other hand the reaction with acetyl chloride took place vigorously to give a dark colored solid which seemed to be a simple addition compound. However, upon extracting an alkaline solution of this solid with ether and removing the solvent from the extract, the solid yielded a colorless oil which proved to be the same even in its optical property as the starting material, XII. 4′-Methylamino-1′-(3-pyridyl)-1′-butanol(XIII)[13] which was obtained by reduction of the oxazine with acetic acid and zinc dust has optical rotatory power; $[\alpha]_D^{24}=-8.690$ in p-dioxane, $[\alpha]_D^{24}=-5.840°$ in 1 N hydrochloric acid.

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[12] A. Pinner and R. Wolfenstein, *Ber.*, **25**, 1428 (1892).

[13] C. H. Rayburn, W. R. Harlan and H. R. Hanmer, *J. Am. Chem. Soc.*, **72**, 1721 (1950).

Reactions analogous to those used for myosmine by Haines, Eisner and Woodward[14] were applied to N′-methylmyosmine. The oxime (VI) of pseudoöxynicotine prepared by the usual method was reduced with acetic acid and zinc dust and the reaction mixture gave five Koenig reaction positive spots ($R_F=0.10$, 0.31, 0.46, 0.57 and 0.85) on a paper chromatogram. The main product proved to be 1′-amino-1′-(3-pyridyl)-4′-methylaminobutane (VII) and the tripicrate melted at 209~210.5°C after recrystallization from water. The hydrazone (VIII) of pseudoöxynicotine, upon treatment with sodium ethoxide in absolute ethanol (a modified Wolf-Kischner's reduction method[15], yielded dihydrometanicotine (IX).

Pyrolysis of N′-methylmyosmine, under reduced pressure, 5 mm., was performed in the range of temperatures from 160°C to above 300°C (bath temperature). At temperatures below 300°C, N′-methylmyosmine derived from 8.1 g of the dihydrochloride of pseudoöxynicotine, yielded a fraction of about one half the weight of the starting material and the fraction contained nicotyrine (X), unchanged N′-methylmyosmine and several other Koenig reaction postitive substances, the latter being revealed by a paper chromatographic study. At temperatures over 300°C (bath temperature) crystals of N′-methylnicotinamide (XI) were deposited on the neck of the distilling flask in a very poor yield. On raising the temperature over the range, a dark brown glassy oil was obtained by distillation and the ultraviolet absorption spectrum of the oil in 0.03 N hydrochloric acid solution exhibited two maxima at 250 and 305 mμ, but further study on this fraction was not successful. Almost half the amount of the starting material remained in the flask as a dark brown oil and became solid on cooling. Oxidation of the solid with potassium permanganate gave a fairly good yield of nicotinic acid. This indicated that N′-methylmyosmine polymerizes without cleavage of the 3-pyridyl configuration.

In the present work, we also succeeded in obtaining nicotine-N′-oxide, in a crystalline form. Goldfarb and Zvorykina[16] reported that they obtained crystalline nicotine-N′-oxide with a melting point of 172~173°C (from acetone). The crystals obtained by us were colorless and melted at 154~155.2°C immediately after recrystallization from ethyl acetate. When these crystals are kept in a desiccator over phosphorus pentoxide for several weeks they become white and then melt at 170~171°C. We presume the lower melting compound must be a hydrated form of the higher melting compound. The latter is hygroscopic, gives an elemental analysis for N which agrees with $C_{10}H_{14}N_2O$, and is optically active, $[\alpha]_D^{21.5}=+65.3$ (c 5.13, methanol). The dipicrate of this compound melts at 169~171°C which agrees with the Russian literature (m.p. 168°C) and gives an elemental analysis which corresponds to $C_{22}H_2N_8O_{15}$.

In addition to these synthetic studies, we reexamined the autoxidation products of nicotine with the hope of proving the presence of N′-methylmyosmine, as an intermediate. The same procedure, as reported previously[10], was employed and careful separation of the oxidation mixture gave N′-methylmyosmine as the dihydrochloride of pseudoöxynicotine in a fairly good yield. This result suggests that N′-methylmyosmine may be an intermediate in the autoxidation process of nicotine and this might also be the case in the fermentation process of tobacco. Futhermore, since we have shown the rearrangement of nicotine-N′-oxide to N′-methylmyosmine under conditions which are more moderate than previously reported, it is altogether possible that nicotine-N′-oxide, which is present during the fermentation process but which has been excluded from the stepwise degradation scheme of nicotine[6], may actually be an intermediate in this degradation. From

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[14] P. G. Haines, A. Eisner and C. F. Woodward, *J. Am. Chem. Soc.*, **67**, 1258 (1945).

[15] D. Todd, "Organic Reactions", Vol. IV, John Wiley & Sons, Inc., New York, N. Y., 1948, p. 378~422.

[16] Ya. L. Gol'dfarb and V. K. Zvorykina, *C. A.*, **52**, 20219 (1959).

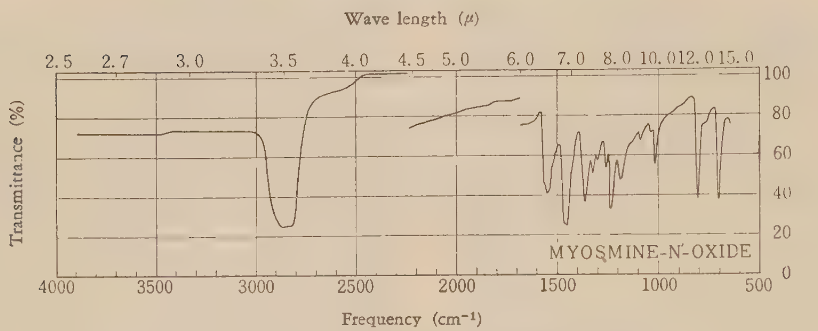

FIG. 1. Infrared Spectrum of Myosmine-N′-oxide.

the study of the chemical properties of N′-methylmyosmine mentioned herein, it seems probable that the base as predicted by Frankenburg et al.[4,6] and by us[10], plays a substantial role in the degradation of nicotine.

A series of reactions similar to those used for the nicotine-N′-oxide synthesis was performed with 10 g of l-nornicotine (XIV). In a 7-day oxidation period with hydrogen peroxide, the nornicotine gave 6 Koenig reaction positive spots on a paper chromatogram. Upon acidifying an aqueous solution of the oxidized mixture, extracting with ether and concentrating the extract, a mixture weighing 1.25 g was obtained. The mixture contained mostly nicotinic acid, m.p. 234°C and a small amount of a crystalline substance which was presumed to be nornicotyrine but further clarification was unsuccessful.

Extraction of the acidic aqueous residue with chloroform and removal of the solvent yielded crude crystals of an anticipated myosmine-N′-oxide (XV) contaminated with a few β-pyridyl compounds. Upon purification on an alumina column, the crude product gave 2.05 g of colorless crystals. The crystals showed no optical activity indicating the disappearance of the asymmetric carbon at the 5′-position in the pyrrolidine ring of nornicotine. The elemental analysis of the compound gave an emprical formula of $C_9H_{10}N_2O$. The infrared absorption spectrum in Nujol exhibited strong absorption bands at 1575 and 1235 cm^{-1} which are attributed to the $>C{=}N-$ and $>N\rightarrow O$ stretching frequencies[17~18] respectively and no absorption bands above the region of 1600 cm^{-1} which indicates the absence of $-OH$, $>NH$ and $>C{=}O$[19]. The ultraviolet absorption spectra of the compound showed maxima at 231 and 285 mμ in water, and 243 and 298 mμ in 0.3 N hydrochloric acid. According to a paper by Swain et al.[20], the presence of the pair of maxima shifted toward the red can be attributed to a double bond conjugated with the pyridine nucleus. The compound was reduced to nornicotine, with acetic acid and zinc dust and dehydrated to nornicotyrine (XVII) with phosphorus pentoxide. Pyrolysis of the compound under vacuum (2 mm) at 130~170°C gave a good yield of myosmine, XVI, accompanied by a trace of nornicotyrine. These results mentioned above indicate that the compound, XV, is myosmine-N′-oxide, [2′-(3-pyridyl)-1′-pyrroline-N′-oxide].

EXPERIMENTAL[21]

Nicotine-N′-oxide (II). By exactly following the

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[17] J. Thesing and W. Sirrenberg, *Chem. Ber.* **92**, 1748 (1959).

[18] H. Shindo, *Chem. & Pharm. Bull.*, **6**, 117 (1958).

[19] See J. L. Bellamy, "The Infrared Spectra of Complex Molecules", Methuen & Co., London, 1958.

[20] M. L. Swain, et al., *J. Am. Chem. Soc.*, **71**, 1341 (1949).

[21] All the melting and boiling points are uncorrected. Ultraviolet absorption spectra were obtained in a Beckman model DU spectrophotometer, and infrared absorption spectra were obtained with a Perkin-Elmer model 21 infrared spectrophotometer. The paper chromatograms were run by the descending technique on Whatman No. 1 filter paper impregnated with 0.2 M sodium acetate in a solvent system of tert. amyl alcohol-acetate buffer, pH 5.6 (1:1, v/v). Microanalysis were performed by Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo, Japan.

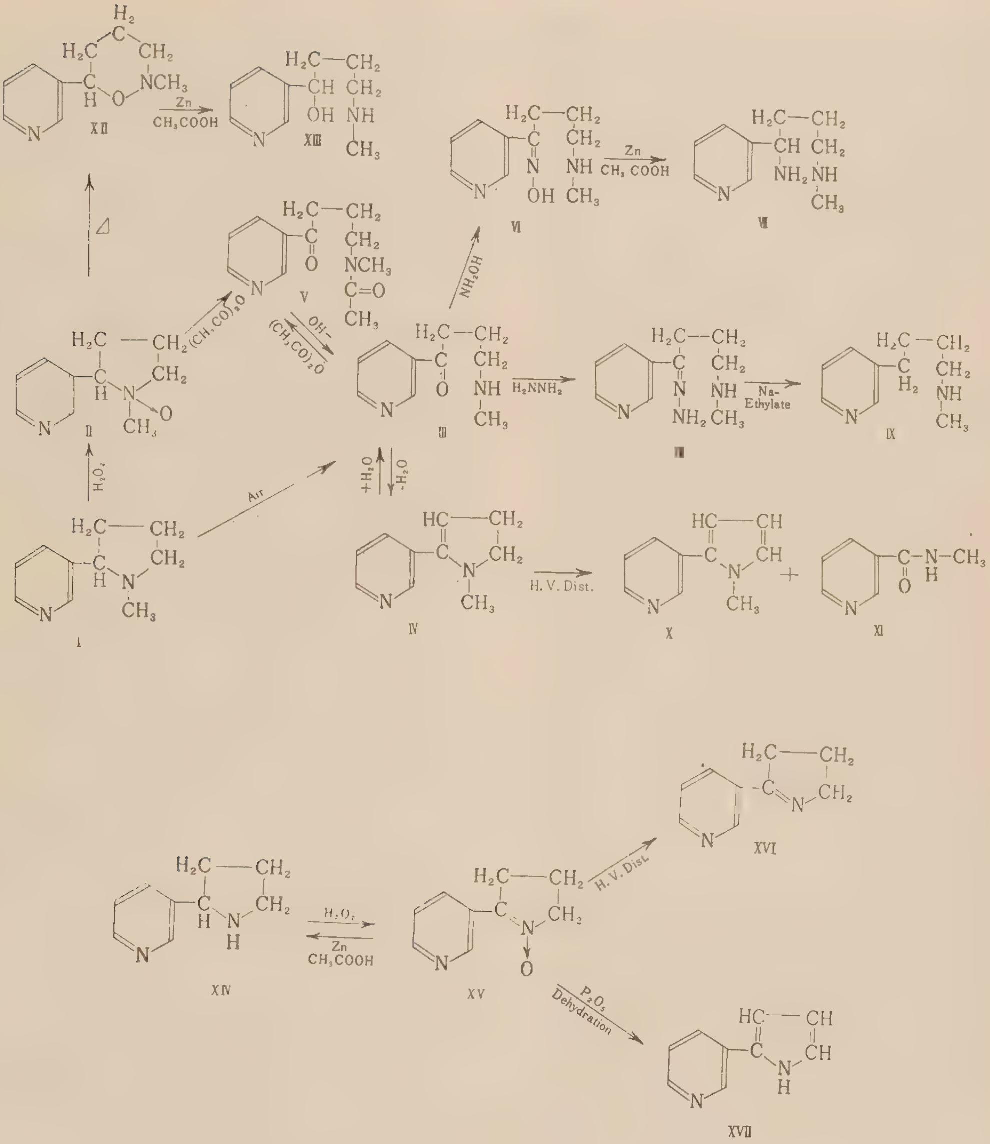

FIG. 2. Transformation of N′-oxides of Nicotine and Nornicotine.

method of Pinner and Wolfenstein[12], 150 g of l-nicotine, b.p. 113°C/9 mm, $[\alpha]_D^{13} = -168.6°$, gave 163 g of glassy nicotine-N′-oxide. The N′-oxide was washed thoroughly with dried ether by stirring and upon repetition of this procedure several times it crystallized as dark brown needles.

Two grams of the crude cyrstals was dissolved in 15 ml of methanol and to the solution was added 5 g of alumina (E. Merck, ♯1097). Then the methanol was evaporated, dried, and the alumina was added on the top of an alumina column (3×8.5 cm). The column was washed with 3 l of ether and was eluted with 300 ml of 5% methanol in ether. The eluate was evaporated to dryness under reduced pressure to yield light yellow needles. The needles were recrystallized twice from ethyl acetate and gave colorless crystals melting at 154~155.2°C. When these crystals are kept in a desiccator over phosphorus pentoxide for several weeks they became white and melted at 170~171°C. $[\alpha]_D^{21}=+65.3°$ (c 5.13, methanol), yield 1.8 g. *Anal.* Calcd. for $C_{10}H_{14}N_2O$: N, 15.72. Found: N, 15.58.

On addition of aqueous saturated picric acid solution, a sample gave an oily picrate which became crystalline on standing at room temperature overnight. A recrystallization from 5% picric acid solution yielded the dipicrate of nicotine-N′-oxide melting at 169~171°C. *Anal.* Calcd. for $C_{22}H_{20}N_8O_{15}$: C, 41.51; H, 3.17; N, 17.61. Found: C, 41.43; H, 3.18; N, 17.67.

1′-(3-pyridyl)-4′-(N′-acetylmethylamino)-1′-propanone (V). Thirty-one grams of crystalline nicotine-N′-oxide was treated with 25 ml of acetic anhydride in small portions while maintaining the reaction temperature below 40°C by external cooling with ice. Another 25 ml of acetic anhydride was added and the mixture was heated at 70°C to reflux for 0.5 hr. The mixture was then made alkaline and extracted three times with chloroform. The combined chloroform extracts, after drying over anhydrous sodium sulfate, were evaporated under reduced pressure to yield a dark red oil. On fractional distillation, the oil afforded a main fraction of 22.15 g boiling at 198~202°C (3.0 mm), which was shown to be 1′-(3-pyridyl)-4′-(N′-acetylmethylamino)-1′-propanone (V), $n_D^{20}=1.5397$. *Anal.* Calcd. for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 64.94; H, 7.10; N, 12.64.

The infrared spectrum of the compound exhibited two strong absorptions at 1680 and 1630 cm^{-1}. The ultraviolet absorption spectra of the compound showed λ_{max} 230 mμ (ε=7400) and λ_{min} 252 mμ (ε=2360) in aqueous solution and λ_{max} 263 mμ (ε=4360) and λ_{min} 241 mμ (ε=1620) in 0.03 N HCl aqueous solution.

One gram of the N-acetylamino ketone (V) in 12 ml of ethanol was refluxed with 2.1 g of 2,4-dinitrophenylhydrazine and 4 ml of concd. hydrochloric acid. The reaction mixture was kept in the refrigerator and yielded 1.83 g of 2,4-dinitrophenylhydrazone hydrochloride, m.p. 213~214°C dec. *Anal.* Calcd. for $C_{18}H_{20}N_6O_5\cdot HCl$: C, 49.48; H, 4.84; N, 19.23; Cl, 8.12. Found: C, 49.25; H, 4.37; N, 19.13; Cl, 8.44.

Pseudoöxynicotine dihydrochloride (III). (a) N-acetylamino ketone (V) (12.59 g) was dissolved in 30% sodium hydroxide solution and steam distilled into a flask containing a small volume of hydrochloric acid. The distillate was concentrated under reduced pressure to yield 14.04 g of yellow needles. The crystals were recrystallized from a mixture of methanol-ethanol (1:6, v/v) and gave colorless needles, m.p. 196~198°C. The melting point was not depressed upon admixing with authentic pseudoöxynicotine dihydrochloride prepared by Pinner and Wolfenstein's method[12]. *Anal.* Calcd. for $C_{10}H_{14}N_2O\cdot 2HCl$: C, 47.82; H, 6.42; N, 11.15. Found: C, 47.57; H, 6.40; N, 11.22.

(b) To 30 g of nicotine-N′-oxide was added 10 ml of acetyl chloride and the mixture was allowed to stand for a while. The reaction was initiated by scrubbing the inside of the container with a glass rod. After the reaction had started evolving heat, a condenser was attached to the flask and the reaction was continued at room temperature for half an hour, during which period about 40 ml of acetyl chloride was introduced in portions. After the reaction period excess acetyl chloride was removed in vacuo and the trace of acetyl chloride remaining in the mixture was decomposed by addition of water under external cooling.

(c) In the same proportions as used above, nicotine-N′-oxide and benzoyl chloride were reacted under ice-bath cooling so as to maintain the reaction temperature below 50~60°C. After an hour, excess benzoyl chloride was decomposed with water and the resultant benzoic acid was removed by extraction with ether after making more acidic with hydrochloric acid.

The reaction mixture of nicotine-N′-oxide either with acetyl chloride or benzoyl chloride as described above was made alkaline under cooling and steam distilled. The distillate was collected in a flask containing dilute hydrochloric acid and upon evaporation to dryness in vacuo it gave reddish crystals. An analytical sample was recrystallized from ethanol and melted at 196~198°C. The yields are summarized in Table I. *Anal.* Calcd. for $C_{10}H_{14}N_2O\cdot 2HCl$: C, 47.82; H, 6.42; N, 11.15. Found: C, 47.94; H, 6.84; N, 11.20.

Pseudoöxynicotine (III) oxime dihydrochloride. Seven grams of pseudoöxynicotine dihydrochloride was dissolved in 15 ml of hot methanol and to the solution was added 2 g of hydroxylamine hydrochloride dissolved in 7 ml of hot methanol.

On keeping it in the refrigerator the mixture yielded 6.89 g of colorless needles, m.p. 205~206°C. Another crop of the oxime was obtained from the filtrate upon addition of 2 volumes of petroleum ether, yield 0.319 g, m.p. 205~206°C. The combined oxime was recrystallized from a mixture of ethanol-methanol (1 : 3, v/v) and afforded 6.6 g of colorless crystals with a constant melting point of 205~206°C dec. *Anal.* Calcd. for

$C_{10}H_{15}N_3O \cdot 2HCl$: C, 45.12; H, 6.44; N, 15.76. Found: C, 45.33; H, 6.37; N, 15.90.

1′-Amino-1′-(3-pyridyl)-4′-methylaminobutane (VII). Six grams of pseudoöxynicotine oxime 2HCl was dissolved in 170 ml of 95% ethanol and to the solution was added 50 ml of glacial acetic acid. Fifty grams of zinc dust was added in small portions with stirring over a period of 9 hrs. and the reaction mixture was allowed to stand at room temperature overnight. The solid was removed by filtration and the funnel was washed thoroughly with ethanol. The filtrate and the washings were combined and they were evaporatively distilled to yield a light yellow syrup as a residue. The residue was taken up in 200 ml of water and the water was removed by vacuum distillation. Another 200 ml of water was added to the residue and 30% sodium hydroxide solution was added until the precipitates which had occurred by initial addition of the sodium hydroxide solution disappered. The alkaline solution was extracted three times with chloroform. The combined chloroform extracts yielded 5 Koenig positive spots ($R_F=0.10$, 0.31, 0.46, 0.57, 0.85), on a paper chromatogram.

Drying and removal of the chloroform left 4.0 g of a light yellow oil which was taken up in 35 ml of water. The solution was made alkaline with 15 ml of 30% sodium hydroxide solution, steam distilled and 350 ml of distillate collected. The distillate contained 3 Koenig reaction positive spots ($R_F=0.46$, 0.57, 0.85) upon chromatography on paper but no further study on this fraction was made at present.

The alkaline solution which had been steam distilled above was adjusted to pH 10.0 and two extractions of the solution with ether removed some impurities. Then the solution was made more strongly alkaline and extracted three times with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and removal of the solvent afforded 1.95 g of 1′-amino-1′-(3-pyridyl)-4′-methylaminobutane (VII) boiling at 129~131°C (3 mm), $n_{\mathrm{D}}^{20}=1.5239$.

Treatment of 0.2 g of the oil with picric acid solution yielded 0.76 g of a tripicrate which melted at 209~210.5°C dec. after are crystallization from water. *Anal.* Calcd. for $C_{28}H_{24}N_{12}O_{21}$: C, 38.82; H, 3.03; N, 19.40. Found: C, 38.77; H, 3.29; N, 19.50.

Dihydrometanicotine (IX). Ten grams of pseudoöxynicotine dihydrochloride and 4 g of anhydrous sodium acetate were suspended in 30 ml ethanol and upon addition of 2.5 g of 80% hydrazine hydrate to the mixture, the reaction took place spontaneously and the color changed to yellow. The reaction mixture was refluxed on a water bath at 70°C for 2 hrs. and then was allowed to stand at room temperature. The mixture was filtered, the filtrate was concentrated in vacuo and the residue was taken up in a minimum volume of water. The solution was made alkaline with 30% sodium hydroxide solution and extracted three times with chloroform. The combined chloroform extracts were dried and evaporated to yield 7.9 g of an orange red viscous oil, which gave a single Koenig positive spot ($R_F=0.22$) on a paper chromatogram. However, purification of the oil by vaccum distillation gave several degraded products.

In a modification of the Wolf-Kischner method[15], a mixture of 7.5 g of pseudoöxynicotine hydrazone obtained as above and 4.0 g of sodium alcoholate in 22 ml of absolute ethanol was heated and the ethanol was distilled off by gradually raising the temperature of the oil bath to 190°C. The mixture was maintained at this temperature for 2 hrs. About 150 ml of water was added after the mixture had been cooled. The aqueous solution was made strongly alkaline and extracted five times with ether. After drying the combined ether extracts with anhydrous sodium sulfate, the solvent was removed by evaporation to yield 6.0 g of oil. The oil was taken up in a small volume of water and subjected to azeotropic distillation to remove some impurities. The distillation residue was made strongly alkaline and extracted five times with ether. The combined ether extracts yielded an orange-colored oil after drying and removed of the solvent. On fractional distillation the oil gave a fraction of 2.7 g boiling at 117~118°C (3 mm) $n_{\mathrm{D}}^{29}=1.5113$. The oil gave a dipicrate which after recrystallization from water melted alone and with authentic dihydrometanicotine picrate at 162°C[22]. *Anal.* Calcd. for $C_{22}H_{22}N_8O_{14}$: C, 42.43; H, 3.56; N, 18.00. Found: C, 42.62; H, 3.79; N, 18.16.

2′-Methyl-6′-(3-pyridyl)-tetrahydro-1′,2′-oxazine (XII). This was prepared by the method of Rayburn et al.[13] Thirty-two grams of nicotine-N′-oxide was vacuum distilled to give a distillate of 32 g boiling at 125~127°C (6mm). The distillate was redistilled and a fraction boiling at 122.5~125°C (4 mm) was obtained. This fraction was presumed to be the oxazine and the picrate melted at 184°C, $[\alpha]_{\mathrm{D}}^{21}=-53.00$ (c 8.11, methanol), $n_{\mathrm{D}}^{20}=1.5340$. *Anal.* Calcd. for $C_{22}H_{20}N_8O_{15}$: C, 41.51; H, 3.17; N, 17.61. Found: C, 41.87; H, 3.21; N, 17.55.

To 5 g of the oxazine (XII) was added 5 ml of acetic anhydride with stirring at room temperature but no reaction took place. The mixture was heated at 70°C on a water bath for 8 hrs. and the color changed to orange. To the reaction mixture was added water, the solution was made alkaline, and extracted with chloroform several times. The dried chloroform extracts were evaporated under reduced pressure and yielded an orange-colored oil which was fractionally distilled in vacuo. A fraction of 2.8 g boiling at 115～118°C (3 mm) was obtained and was proved to be identical to the starting material, XII, by infrared spectrophotometry.

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[22] K. Löffler and S. Kober, *Ber.*, **42**, 3431 (1909).

Under ice-bath cooling, 5 ml of acetyl chloride was added dropwise to 3.7 g of the oxazine (XII) and when one-third the volume of the chloride had been added an exothermic reaction occurred. After the vigorous reaction ceased, the mixture was refluxed at 70°C for half an hour during which period the mixture deposited a resinous mass.

After removal of excess acetyl chloride by vacuum distillation the reaction mixture was dissolved in 20 ml of water and made strongly alkaline under cooling. The solution was extracted with chloroform several times and the combined chloroform extracts, after drying, were evaporated to yield a dark reddish viscous oil, yield 3.14 g. Upon fractional distillation under vaccum the oil yielded a colorless oil of 1.6 g boiling at 115～117°C (3 mm), $[\alpha]_D^{21}=-50.95$. The infrared spectrum showed that the oil was identical to the starting material, XII.

4′-Methylamino-1′-(3-pyridyl)-1′-butanol (XIII). Exactly following the procedure reported by Rayburn et al.[13], 6 grams of the oxazine was dissolved in 32 ml of 10% acetic acid and 6 g of zinc dust was added in small portions with stirring. The reaction mixture gave a light yellow, viscous oil; yield 1.3 g; b.p. 183～186°C (6.5 mm). The optical rotations of the oil were $[\alpha]_D^{24}=-8.690°$ (c 5.902, *p*-dioxane) and $[\alpha]_D^{24}=-5.840°$ (c 6.503, 1 N HCl aq. soln.). The dipicrate melted at 137～138°C.

Isolation of pseudoöxynicotine from autoxidation products of nicotine. This work was especially conducted to establish the presence of pseudoöxynicotine in the autoxidation products of nicotine. Thirty-five grams of freshly distilled nicotine, b.p. 107°C (7.5 mm), was gently aerated for 5 months under the same conditions as described in a previous paper[10]. After the aeration period, 400 ml of water was added to the dark brown oxidation mixture which weighed 39 g and the presence of pseudoöxynicotine ($R_F=0.11$) in the mixture was revealed paper chromatographically. The resulting solution was adjusted to pH 7.3 with hydrochloric acid and sodium bicarbonate. The solution was transferred into a liquid-liquid extractor and extracted with ether for 50 hrs. to remove nicotyrine and unchanged nicotine, which together weighed 23.7 g.

The aqueous solution was made acidic with hydrochloric acid and concentrated to 50 ml in vacuo. The residue was made strongly alkaline with sodium hydroxide solution and steam distilled. The distillate was collected in two fractions of 250 ml and 4 l each in flasks containing dilute hydrochloric acid. On evaporation of the water under reduced pressure, both fractions yielded a glassy oil. After addition of a small volume of ethanol and allowing to stand at room temperature, both residues deposited crystals. The former fraction yielded 0.216 g of very hygroscopic needles and the latter gave 0.321 g of tiny powder-like crystals. From the infrared absorption spectrum, the former crystals were presumed to be a mixture of the dihydrochlorides of nicotine and pseudoöxynicotine. After several recrystallizations from ethanol, the latter yielded 30 mg of white crystals, m.p. 196～197°C dec. which was not depressed on admixing with authentic pseudoöxynicotine dihydrochloride. *Anal.* Calcd. for $C_{10}H_{14}N_2O\cdot 2HCl$: C, 47.82; H, 6.42; N, 11.15. Found: C, 47.82; H, 6.54; N, 11.17.

Pyrolysis of N′-methylmyosmine. Eight and one-tenth grams of pseudoöxynicotine dihydrochloride in 150 ml of water was made strongly alkaline and extracted twice with chloroform. N′-methylmyosmine was obtained by removal of the solvent and was vacuum distilled directly at 5 mm to yield two fractions (A and B).

Fraction A was collected up to 126°C (bath temperature 160～180°C). Fraction B consisted of material which crystallized in the distilling tower and side arm when the bath temperature was raised over 300°C. The crystals were washed out with chloroform. The residue in the distilling flask was further distilled by raising the temperature over the range and a dark brown oil was obtained. Only its absorption in the ultraviolet was investigated.

Fraction A weighed 2.6 g and was taken up in a small amount of water, which was adjusted to pH 4.5 with dilute hydrochloric acid and extracted with ether several times. After drying and removal of the solvent, the residue of the ether extract was taken up in water, made alkaline and then steam distilled. The distillate was made alkaline, extracted with ether several times, and the combined ether extracts, on removal of the solvent, yielded 13 mg of oil. Treatment of the oil with aqueous picric acid yielded a picrate which after two recrystallizations from water melted at 168～170°C alone and with authentic nicotyrine monopicrate. *Anal.*

Calcd. for $C_{16}H_{13}N_5O_7$: C, 49.62; H, 3.37; N, 18.08. Found: C, 49.78; H, 3.22; N, 17.65.

The aqueous residue of the first ether extraction at pH 4.5 was adjusted to pH 10.0 with alkali and extracted with ether. The combined ether extracts were evaporated to yield 0.7 g of oil which was then subjected to azeotropic distillation. By the usual method the distillate gave 12.2 mg of oil which was proved to be nicotine by its picrate, m.p. 223°C, alone and with an authentic sample.

Fraction B yielded 103 mg of crystals which were taken up in a minimum amount of chloroform and chromatographed on an alumina column. The column was eluted with ether and 5% methanol in ether and a fraction having a positive Koenig reaction was collected. The fraction was concentrated in vacuo to dryness and the residue was recrystallized from benzene several times to yield 21.6 mg of N′-methylnicotinamide, m.p. 102~103°C. The mixed melting point with an authentic sample was not depressed. The infrared and ultraviolet absorption spectra of the sample were identical with those of N′-methylnicotinamide. *Anal.* Calcd. for $C_7H_8N_2O$: C, 61.75; H, 5.92; N, 20.58. Found: C, 61.63; H, 5.53; N, 20.38.

Preparation of myosmine-N′-oxide. By following a similar method to that used for the nicotine-N′-oxide preparation[12), a mixture of 10 g of l-nornicotine, $[\alpha]_D^{20}=-45.5°$, isolated in a previous paper[23), 30 g of 30% hydrogen peroxide solution and 250 ml of water was allowed to stand at room temperature for a week. At the end of this time the remaining hydrogen peroxide was decomposed by the addition of manganese dioxide, and the solution was filtered. The filtrate was adjusted to pH 3.5 with dilute hydrochloric acid (1:4) and extracted with ether and then with chloroform (4.5 l in total). The combined chloroform extracts were evaporated to yield 5.5 g of crystals, which gave 4.1 g of light brown crystals when chromatographed on an alumina column, using 5% methanol in ether as the eluting solvent. They were recrystallized from pertoleum ether three times and yielded 2.05 g of colorless crystals, m.p. 90~91°C, $[\alpha]_D^{21}=0°$ (c 1.7, methanol). *Anal.* Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.22; N, 17.27. Found: C, 66.50; H, 6.20; N, 17.33.

The ultraviolet absorption spectra of the compound in water and 0.3 N hydrochloric acid solution showed λ_{max} 231 mμ (ε=8530) and 285 mμ (ε=10850) and λ_{min} 246 mμ (ε=4350), and λ_{max} 298 mμ and 243 mμ and λ_{min} 258 mμ (ε=2490), respectively. The infrared absorption spectrum showed strong absorption bands at 1575 and 1235 cm^{-1} and the absence of any absorption above the region of 1600 cm^{-1}.

Treatment with aqueous picric acid solution yielded a dipicrate, m.p. 175~176°C dec.

Nornicotine from the N′-oxide. To a stirred solution of 117.2 mg of the N′-oxide in 20 ml of 30% acetic acid was added 0.5 g of zinc dust in portions. The mixture was allowed to stand at room temperature for 5 hrs. and then filtered. The filtrate was made strongly alkaline and extracted with chloroform several times. The combined chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield 31.6 mg of oil. The ultraviolet and infrared absorption spectra were identical with those of nornicotine.

Treatment of the oil with aqueous picric acid solution yielded a dipicrate melting at 187°C and the melting point showed no depression on admixing authentic nornicotine dipicrate (m.p. 190°C).

Nornicotyrine from the N-oxide. To a solution of 48.3 mg of the N′-oxide in 75 ml of xylene was added 0.5 g of phosphorus pentoxide and the mixture was refluxed for 1 hour. After cooling excess phosphorus pentoxide was decomposed by addition of ice-flakes. The solution was made alkaline and extracted with ether several times. The dried ether extract, upon evaporation, gave 18.7 mg of nornicotyrine. A picrate was prepared by the usual method and melted at 199°C, alone and with authentic nornicotyrine picrate[24).

Pyrolysis of the N′-oxide. Under vacuum distillation at 5 mm, 169.1 mg of the N′-oxide yielded a 151.4 mg fraction containing partly solidified particles. The oil was dissolved in a small amount of water, adjusted to pH 3.5 and extracted with ether several times. Drying and evaporation of the combined ethereal extracts yielded nornicotyrine which was recrystallized from ligroin, m.p. 100~102°C, yield 9.3 mg. The melting point was not altered on mixing with authentic nornicotyrine[23). The picrate melted at 198~201°C. *Anal.* Calcd. for $C_9H_8N_2$: C, 74.97; H, 5.59; N, 19.43. Found: C, 74.63; H, 5.44; N, 19.29.

Extraction of the acidic aqueous residue with chloroform, and removal of the solvent, yielded 24.1 mg of unchanged myosmine-N′-oxide, m.p. 80~81°C.

The acidic residual solution was then neutralized, extracted with chloroform several times and the combined extracts were evaporated to dryness. The residue was chromatographed on an alumina column and the ether

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23) E. Wada, *Arch. Biochem. Biophys.*, **62**, 471 (1956).

24) M. Ehrenstein, *Arch. Pharmazie*, **269**, 627 (1931).

eluate, upon evaporation, yielded 60.3 mg of crude crystals. They were dissolved in a minimum volume of petroleum benzin and upon chilling at −10°C the solution deposited crystals. Repetition of the procedure several times afforded 40.4 mg of myosmine m.p. 44.5°C *Anal*. Calcd. for $C_9H_{10}N_2$: C, 73.97; H, 6.85; N, 19.18. Found: C, 74.24; H, 6.77; N, 18.70.

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Enzymatic Degradation of Lignin

Part I. Paperchromatographical Separation of Intermediate Degradation Products of Lignin by the Wood-rotting Fungns *Poria subacida* (Peck) Sacc.

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The intermediates of biological degradation of lignin by *Poria subacida* (Peck) Sacc. were detected by paperchromatography. *p*-Hydroxy-*m*-methoxyphenylpyruvic acid was identified as its 2,4-dinitrophenylhydrazone in the methylenchloride extract of the culture filtrate of the medium, in which spruce native lignin was contained. Guaiacylglycerol-β-coniferylether and vanillic acid-like compound were recognized in acetone extracts and methylenchloride extract of the decayed spruce-wood meal.

The decayed lignin extracted from the decayed wood-meal showed the remarkable variation and the existence of the more predominant amount of carbonyl groups, compared with native lignin on their ultraviolet absorption spectra and infrared absorption spectra, and gave no purple-red colour reaction with phloroglucin-hydrochloric acid reagent.

INTRODUCTION

Biochemical degradation of lignin by microorganisms is of special significance not only on the formation of humus from lignin in the soil of forest, but also on the utilization of lignins which are contained in the waste liquor of paper-industry, and many studies were reported in relation to these problems. On the former, Lindeberg[1] and Fischer[2] reported the biological degradation of lignins by soil *Hymenomycetes* and soil bacteria, and on the latter, Gottlieb and his coworkers[3~5] and Kleinert and Joyce[6,7]

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1) G. Lindeberg, *Z. Pflanzenernähr. Düug Bokenk.*, **69**, 142 (1955).

2) G. Fischer, *Arch. Microbiol.*, **18**, 397 (1953).

3) S. Gottlieb and J. Pelczar, J. R., *Bacteriol. Revs.* **15**, 55 (1951).

4) M. J. Pelzar, S. Gottlieb and W. C. Day, *Arch. Biochem.* **23**, 360 (1949), *ibid.*, **25**, 449 (1950).

5) S. Gottlieb, *Phytopathology*, **40**, 926 (1950)

6) T. N. Kleinert and C. S. Joyce, *Palp and Paper Mag. Can.*, **56**, 206 (1955).

7) T. N.K leinert and C. S. Joyce, *Svenk Papp.*, **62**, 37 (1959).

studied on the wood-rotting fungi or fungi and bacteria which have the ability to decompose lignins.

In spite of many investigations on these problems, up to today, no certain evidence has been obtained on the mechanisms of the degradation of lignin by the microorganisms and its enzymic action concerned. Gottlieb and Pelczar[3] pointed out that a confusing array of data pertaining to the action of bacteria, fungi and enzymes on lignin which appeared in the past literature, depended on the lack of definite knowlege of the structure and properties of lignin, and Nickerson[8] described that these confusion may have been occured by the deficiency of the experimental techniques.

Gottlieb[5] attempted a simultaneous adaptation of *Poria subacida* and *Polyporus abietinus* to growth in various lignin residues, and showed that these wood-rotting fungi grew on liquid media containing various lignin materials as "limiting source of carbon". Gottlieb and Geller[9] succeeded to obtain a crude enzyme powder from the pressed juice of commercial mushroom spawn (*Agricus campertris*) which oxidised native lignin. Van Vliet[10] cultivated the white-rot fungi *Poria subacida* and *Coriolus versicolor* on an inorganic medium with native lignin as a sole carbon source, and showed that enzyme preparation from mushroom and the culture filtrates of *Coriolus versicolor* oxidised Brauns lignin slowly and hydroquinone, *p*-phenylendiamine rapidly. Konetzka, Pelczar and Gottlieb[11] isolated *Flavobacterium* sp. which is capable of utilizing α-conidendrin as a sole carbon source. Higuchi, I. Kawamura and H. Kawamura[12] studied the properties of lignin in decayed wood, and showed that coniferylaldehyde, vanillin and syringaldehyde could be found from ethanol-benzene extract of decayed beech-wood meal attracked by several white-rot fungi and suggested that "the open vanillin or syringaldehyde yielding elements of the lignin" by nitrobenzene oxidation were preferentially attacked during the fungal destruction of lignin.

Fukuzumi, Minami and Shibamoto[13~15] attempted the investigation on the oxidation of some simple aromatic compounds by the white-rot fungus, *Polystictus sanguineus* and researched the metabolic products by the fungus in the medium, which contained benzoic acid as a sole carbon source, and caught acetaldehyde, acetoacetic acid and α-ketoglutaric acid.

For the purpose of pursuing the mechanisms and enzyme action of biological degradation of lignin, the use of the living cell is not suitable because intermediate products are hardly accumulated but may by further metabolised, and therefore enzymatic study must be adopted. Direct oxidation of lignin by enzyme is weak according to the experiments by Higuchi[16] and Van Vliet[10], probably because of a high molecularity of lignin.

Quite recently, the author[17] studied the phenol oxidase activity of the enzyme in the culture filtrate of *Poria subacida* and found that the mixed enzymes of *Poria subacida* oxidised *p*-hydroxy-*m*-methoxyphenylpyruvic acid, a hypothetical intermediate of biological degradation of lignin, and liberated carbon dioxide.

In this report, the intermediates of biological degradation of lignin by *Poria subacida* are studied by the method of paperchromatography, and the results support the author's previous opinion[17], that lignin is decomposed via guaiacylglycerol-β-ether compounds, β-oxyconiferylalcohol and *p*-hydroxy-*m*-methoxyphenylpyruvic acid by the enzymes of *Poria subacida.*

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[8] W. J. Nickerson, *Ind. Eng. Chem.*, **48**, 1411 (1956).

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[16] T. Higuchi, *Jour. Jap. Forest. Soc.*, **35**, 77 (1953).

[17] T. Fukuzumi, *Jour. Jap. Wood Res. Soc.*, **5**, 222 (1959).

EXPERIMENTAL AND RESULTS

1. Culture filtrate of *Poria subacida*, in the medium of which, lignin, xylose and asparagine are contained.

Medium: Nord's native[18] lignin, which was extracted from wood-meal (*Picea jezoensis* Carr.) decayed by the brown-rot fungus *Poria vaporaria* by the same method as in the previous paper[17], 1 g; xylose 5 g; asparagine 2.5 g; yeast 2 g water extract; basal medium (described in the previous paper[17]), were made to 1 l and adjusted pH to 6.0.

3 l of this medium was devided to 100 ml in 500 ml shaking flasks and sterilized at 115°C for 20 minutes. Mycelial pellets suspension of *Poria subacida* were inoculated in this culture medium and shaken at 26~27°C for 5 days and then stayed in an incubator at 26°C for 15 days.

This culture medium was filtered, and ammonium sulfate was added in the filtrate to saturation. Then the filtrate was extracted three times with total 500 ml of methylenchloride, further extracted with ether at pH 2.0 H_2SO_4 acidity. Each extract was evaporated under nitrogen current and mild suction to a small volume and used to the chromatographical experiment.

TABLE I. R_F VALUE OF THE EXTRACTS OF THE CULTURE FILTRATE

Solvent	Butanol, ethanol, water=5:1:4				Butanol, acetic acid, water=4:1:5			
Spraying reagent	Diazobenzenesulfanilic acid		2,4-Dinitrophenylhydrazine		Diazobenzenesulfanilic acid		2,4-Dinitrophenylhydrzine	
R_F value, Colour / Sample	R_F value	Colour	R_F value	Colour	R_F value	Colour	R_F value	Colour
Methylenchloride ext.	0.88	light orange	0.85	yellow orange	0.84	orange	0.86	yellow orange
Ether ext. from above ext.	—	—	—	—	—	—	—	—
p-OH-*m*-OCH_3 Phenylpyruvic	0.88	red orange	0.85	orange	0.84	light orange	0.86	yellow orange
p-OH Phenylpyruvic acid	0.90	light orange	0.86	brown	0.86	purple orange	0.88	brown

1) Paperchromatography of the extracts.

Samples were developed on the papers (Toyo filter-paper No. 50 2×40 cm) by the following solvents[19]: a) butanol-ethanol-water (5:1:4), b) butanol-acetic acid-water (4:1:5), for 18~20 hours. R_F values, which were indicated by the spraying reagent: diazobenzenesulfanilic acid and sodium carbonate solution of 0.1% 2,4-dinitrophenylhydrazine, hydrochloric acid methanol water solution are shown in Table I.

2) Paperchromatography of 2,4-dinitrophenylhidrazone of the extracts.

2,4-Dinitrophenylhydrazones of *p*-hydroxy-*m*-methoxyphenylpyruvic acid, *p*-hydroxyphenylpyruvic acid, 3,4-dihydroxyphenylpyruvic acid and the methylenchloride extract were prepared by means of the method of Shriner[20]. These 2,4-dinitrophenylhydrazones were dissolved in pH 7.0, 1/10 M phosphate buffer, and spoted on the paper and dried by blower, and then developed for 15 hours by the solvent[21]; tertiary amylalcohol-ethanol-water (5:1:4). R_F values of the hydrazones are shown in Table II. Standard phenylpyruvic acid derivatives were synthesized by the method of "Organic synthesis Vol. 27, P. 890, phenylpyruvic acid." These yellow spots of 2,4-dinitrophenylhydrazones turned brown on ammonium vapour.

TABLE II. R_F VALUE OF THE HYDRAZONES

Solvent; Ter. $C_5H_{11}OH$, EtOH, H_2O=5:1:4

Hydrazones	R_F value
Methylenchloride extract	0.75
p-OH-*m*-OCH_3 Phenylpyruvic acid	0.75
p-OH Phenylpyruvic acid	0.77
3,4-Dihydroxyphenylpyruvic acid	0.74

The R_F value of methylenechloride extract and its 2,4-dinitrophenylhydrazone did agree well with those of *p*-hydroxy-*m*-methoxyphenylpyruvic acid.

According to the above chromatographycal experiments, it was confirmed that *p*-hydroxy-*m*-methoxyphenylpyruvic acid was contained in the methylenchloride extract of the culture filtrate in which lignin was decomposed by *Poria subacida*.

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[21] S. M. Altmann, E. M. Crook and S. P. Datta, *Biochem. J.*, **49**, lxiii (1951).

2. Wood-meal decayed by *Poria subacida*.

500 g of the wood-meal (*Picea jezoensis* Carr.) which was treated with dilute hydrochloric acid solution and extracted with acetone-water as mentioned in the previous paper[22], was added with 2.5 l of Nord's nutrients: peptone 1.0 g, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 1.0 g, thiaminhydrochloride 2.0 mg per 1 l, adusted pH to 5.6, and was softly packed in the 10 bottles (for 500 g commercial reagent) with 1.5 cm dia. hole of vacant space. Then these culture-bottles were sterilized by intermittent method, and mycelial pellets suspension of *Poria subacida* were inoculated into the bottles.

After these culture-bottles were incubated for 6 months (No. 1) or 12 months (No. 2) at 26°C, the content of the decayed wood-meal was scraped out, and packed into the 10 l bottle, and then added with 1/100 M potassium phosphate buffer (pH 6.5). This bottle, was shaken for 3 hours at room temperature, and then the content was filtered through a large buchner funnel. The filtrate (Filtrate A) was saturated with ammonium sulfate and extracted with methylenchloride, acidified with H_2SO_4 to pH 2.0 and extracted with ether successively. These extracts were concentrated to small volume by evaporation under mild suction and nitrogen current, and used for the chromatographical experiment.

The residual wood-meal was covered with potassium phosphate buffer (pH 6.5), added over its surface with 100 ml of toluen and stored for two days at room temperature (No. 1) or a month at 5°C (No. 2).

Then the content was filtered, and the filtrate (Filtrate B) was extracted with methylenchloride and with ether in acidity successively, evaporated to small volume as mentioned above, and used for the chromatographycal experiment.

Further, the residual wood-meal was extracted with acetone, and acetone of the extract solution was distilled off under nitrogen current and mild suction. The residual water solution was brought to half saturated with ammonium sulfate, and the precipitate was gathered on a buchner funnel, and the filtrate was extracted with methylenchloride, and with ether successively. These extracts (Fraction C) were concentrated to small volume and used for the chromatographical experiments.

Method of paperchromatography.

Filterpaper: The Toyo filterpaper No. 50 (2×40 cm).

Developing solvents: Butanol-ethanol-water (5:1:4 vol.), ascending method[19]. Water saturated with benzene, descending method[23]. Dimethylformamide-tetrachlorocarbon (2:9 vol.), descending method[24].

Spraying reagent: Diazobenzenesulfanilic acid and sodium carbonate solution for guaiacyl derivatives. 0.1% 2,4-Dinitrophenylhydrazine-hydrochloric acid methanol water solution for carbonyl compounds. 0.1% 2,6-Dichloroindophenol ethanol solution[25] for acids. Quinonemonochloroimide[26] solution and sodium carbonate solution for benzylalcohol groups.

Folin and Donis solution[27] for phenolic compounds.

The results of paperchromatography are shown in Table III for No. 1 decayed wood-meal and in Table IV for No. 2 decayed wood-meal.

Standard sample used for the paperchromatography.

Vanillic acid: Synthesized by the method of "Organic synthesis, **30**, p. 101, i. silver oxide method." white needles, m.p. 207°C.

Guaiacylacetone: Student Michio Terasawa synthesized this substance by the method of Hibbert[28]. yellow oil, b.p. 115°C under 0.15 mmHg.

α-Hydroxypropiovanillon: Kindly supplied by Professor Dr. Karl Freudenberg, Forschungsinstitut Freudenberg Heidelberg, Germany.

Lignin constituent intermediate: Kindly supplied by Professor K. Freudenberg. A=Dehydrodiconiferylalcohol, B=Pinoresinol, C=Guaiacylglycerol-β-coniferylether.

Ultraviolet absorption spectrum of the spots of paperchromatography.

At the experiment of paperchromatography using dimethylformamide and tetrachlorocarbon as solvent, the spots corresponding to R_F value 0.14 and 0.36 (Table III 3) were cut off and extracted with each 5 ml of 95% ethanol. The extract solutions were used for ultraviolet spectral analysis (Fig. 1, Fig. 2). The absorption curves were so drawn as the maximum extinction coefficient of the sample coinciding with that of corresponding authentic compound.

The ultraviolet absorption spectrum of the solution of

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[22] T. Fukuzumi and T. Shibamoto, *Jour. Jap. Wood Res. Soc.*, **4**, 15 (1958).

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TABLE III. R_F VALUE OF THE EXTRACTS OF FILTRATE OF DECAYED WOOD-MEAL (No. 1)

1) Solvent, Butanol, Ethanol, Water = 5 : 1 : 4

Spraying reagent / R_F value, Colour / Sample	Diazobenzenesulfanilic acid and Na_2CO_3		2,4-Dinitrophenylhydrazine-HCl		2,6-Dichloro-indophenol	
	R_F value	Colour	R_F value	Colour	R_F value	Colour
Fraction A ether ext.	0.80	orange	0.84	yellow	0.80	pink
p-OH-*m*-OCH_3-Phenylpyruvic acid	0.83	red orange	0.83	orange yellow	0.80	pink
p-OH Phenylpyruvic acid	0.84	orange	0.84	brown	0.84	pink
Fraction B Methylenchloride ext.	0.80	brownred	0.80	yellow	—	—
Fraction B ether ext.	0.82	red	0.84	yellow	0.80	pink
p-OH-*m*-OCH_3-Phenylpyruvic acid	0.82	red	0.85	yellow	0.84	faint pink
p-OH Phenylpyruvic acid	0.88	orange	0.86	brown yellow	0.85	faint pink

2) Solvent; Water saturated with benzene

Spraying reagent / R_F value, Colour / Sample	Diazobenzenesulfanilic acid and Na_2CO_3		Quinonemonochloroimide and Na_2CO_3		Ultraviolet light
	R_F value	Colour	R_F value	Colour	Colour
Fraction B methylenchloride ext.	0.64	orange			dark
	0.75	yellow			
	0.82	faint orange			
Fraction B ether ext.	0.65	orange			
	0.90	brown			
Vanilin	0.64	orange			dark
Guaiacylacetone	0.85	pink			
Fraction C methylenchloride ext.	0.47	orange	0.45	blue	
	0.67	pink	0.65	blue	

3) Solvent; Dimethylformamide, Tetrachlorocarbon = 2 : 9

Spraying reagent / R_F value, Colour / Sample	Diazobenzenesulfanilic acid and Na_2CO_3		Quinonemonochloroimide and Na_2CO_3	
	R_F value	Colour	R_F value	Colour
Fraction C methylenchloride ext.	0.14	yellow	0.14	blue
	0.36	orange	0.36	blue
	0.68	faint orange	0.72	faint blue
Vanilic acid	0.38	orange	0.38	blue
Lignin constituent intermediate* C	0.14	yellow	0.14	blue
A	0.28	red		
B	0.70	red		

* A=Dehydrodiconiferylalcohol B=Pinoresinol C=Guaiacylglycerol-β-coniferylether

R_F value 0.14 (Fig. 1) had a minimum at 241~245 mμ and a maximum at 269~274 mμ, which are almost identical with the minimum and maximum point of the absorption spectrum of guaiacylglycorol-β-coniferylether reported by Freudenberg and Eisenhut[29], and the shape of the absorption spectrum curve also resembled that of above substance.

The ultraviolet absorption spectrum of the solution of R_F value 0.36 is shown in Fig. 2, which resembles that of vanillic acid (Fig. 3) and the shape of coniferylalcohol reported by Freudenberg and Schumacher[30].

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[29] K. Freudenberg und W. Eisenhut, *Chem. Ber.*, **88**, 626 (1955).

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TABLE IV. R_F VALUE OF THE EXTRACTS OF FILTRATE OF DECAYED WOOD-MEAL (No. 2)

1) Solvent; Water saturated with benzene

Spraying reagent	Ultraviolet light		Diazobenzene-Sulfanilic acid Na_2CO_3		Folin and Denis reag., Na_2CO_3		2,4-Dinitro-phenylhydrazine-HCl	
Sample \ R_F value, Colour	R_F value	Colour	R_F value	Colour	R_F value	Colour	R_F value	Colour
Fraction A methylenchloride ext.	0.63	bright blue	0.37	orange	0.40	greyblue	0.48	yellow
			0.63	faint orange	0.65	blue		
Fraction A ether ext.	0.62	dark	0.42	orange	0.41	greyblue		
			0.52	faint orange				
			0.62	orange	0.65	blue	0.62	yellow
α-Hydroxypropiovanillon					0.68	blue		
Vanillin	0.63	dark	0.63	orange			0.63	blue
Lignin constituent intermediate A			0.40	red	0.40	blue		
B			0.50	red	0.50	blue		
C			0.60	yellow orange	0.60	blue		

2) Solvent; Dimethylformamide, tetrachlorocarbon=2:9

Sample	R_F value	Colour	R_F value	Colour	R_F value	Colour
Fraction A methylenchloride ext.	0.1~0.60	yellow blue	0.16	yellow	0.15	blue
			0.28	yellow orange	0.60	blue
			0.45	yellow orange		
Fraction A ether ext.	0.08	yellow	0.08	faint yellow	0.13	blue
	0.00	yellow	0.30	yellow	0.35	blue
					0.52	blue
Lignin constituent intermediate C			0.16	yellow	0.16	blue
A			0.37	red	0.37	blue
B			0.76	red	0.76	blue

3) Solvent; Dimethylformamide, tetrachlorocarbon=2:9

Spraying reagent	Diazobenzenesulfanic acid and Na_2CO_3		Quinonemonochloroimide and Na_2CO_3	
Sample \ R_F value, Colour	R_F value	Colour	R_F value	Colour
Fraction C methylenchloride ext.	0.48	orange	0.14	green blue
			0.45	blue
Fraction C butanol ext.	0.45	faint orange	0.14	green blue
			0.43	green blue
Lignin constituent intermediate C	0.14	yellow orange	0.14	blue
A	0.28	red		
B	0.70	red		

The original methylenchloride extract of Fraction C was further separated by passing through the column of cellulose powder with the solution of dimethylformamide-tetrachlorocarbon (3:9 vol.), and the fraction corresponding to the above substance (R_F 0.36) was again developed on the paper with the solvent, dimethylformamide-tetrachlorocarbon (2:9 vol.). The substance in the fraction showed the same R_F value 0.38, colour reaction with diazobenzene-sulfanilic acid and quinonemonochloroimide with those of vanillic acid. Ultraviolet absorption spectrum of the ethanol solution of the substance and the spot occupied area R_F value 0.38, (Fig. 3), almost resembled that of vanillic acid, but its maximum 252~254 mμ was a little shorter than the

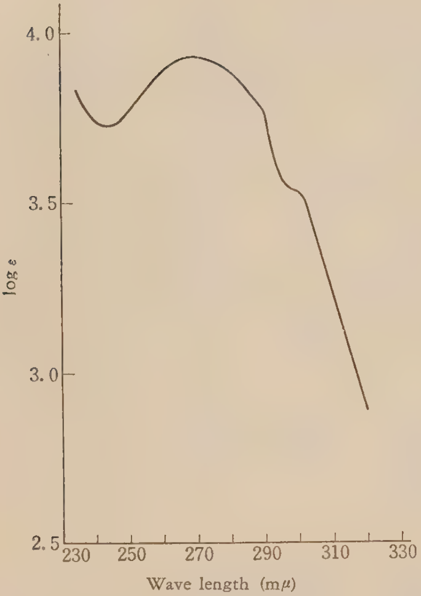

FIG. 1. Ultraviolet Absorption Spectrum of the Corresponding Compound with Guaiacylglycerol-β-coniferylether.

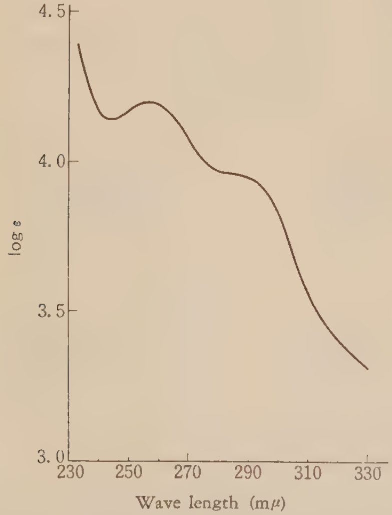

FIG. 2. Ultraviolet Absorption Spectrum of the Substance R_F 0.36 (in Table III 3), Drawn as the max. ε coincides with that of Coniferylalcohol.

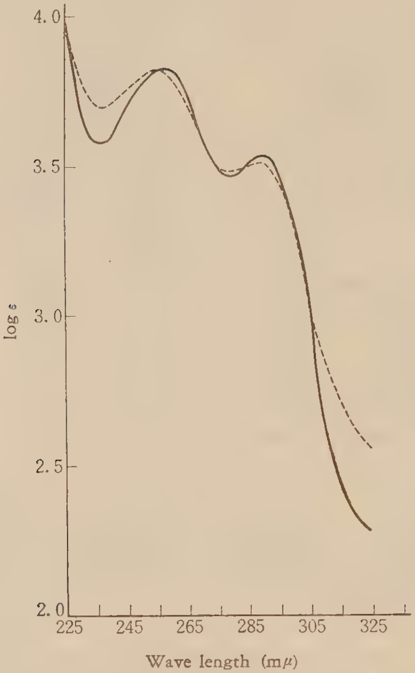

FIG. 3. Ultraviolet Absorption Spectrum of the Corresponding Compound with Vanillic acid.

---- Corresponding compound
—— Vanillic acid

maximum 256~258 mμ of the spectrum of vanillic acid, and the another paperchromatographical experiment useing the solvent, water saturated with benzene, showed R_F value 0.80 for this substance and R_F value 0.90 for vanillic acid. Therefore, it is presumed that this substance is not identical with vanillic acid but its chemical constraction is similar to vanillic acid.

The spectral analysis of decayed lignin.

Decayed lignin: The precipitate, which was obtained from the acetone extract of the decayed wood-meal, was dissolved in acetone-water (9:1 vol.), and the solution poured into ten volume of benzene and the resulted precipitate was dried. The dried precipitate was dissolved in ethylenchloride-ethanol (2:1 vol.) and poured into ten volume of ether. The resulted precipitate was dried in the vacuum desiccator and used for ultraviolet and and infrared spectral analysis.

Infrared spectrum of decayed lignin and Nord's native lignin were obtained by the method of Disk (3 mg lignin and 600 mg of KBr were mixed and pressed to a trancеparent tablet). The spectra (Fig. 4 and Fig. 5) show the existence of a large amount of carbonyl group at the wavenumber 1700 cm^{-1} in decayed lignin compared with that of Nord's lignin. This carbonyl group in decayed lignin may be attributed to ketone group and

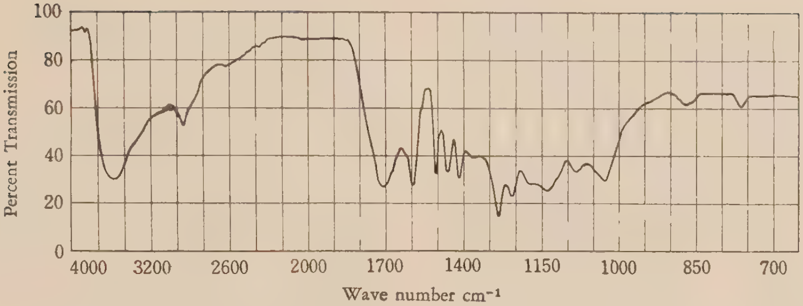

FIG. 4. Infrared Absorption Spectrum of Decayed Lignin (*Picea jezoensis* Decayed by *Poria subacida*).

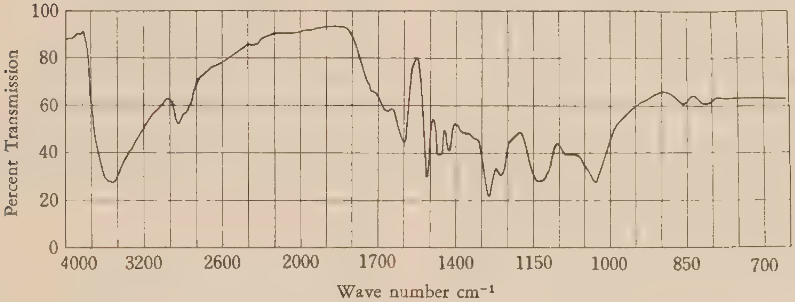

FIG. 5. Infrared Absorption Spectrum of Nords' Lignin (*Picea jezoensis*).

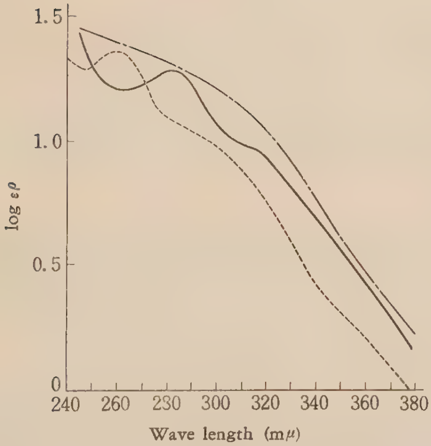

FIG. 6. Ultraviolet Absorption Spectrum of Nords' Lignin (*Picea jezonsis*) and Decayed Lignin (*Picea jezonsis* Decayed by *Poria subrcida*).

—— Nords' lignin
---- Freshly prepared decayed lignin
·—· Decayed lignin (some time after preparation)

not to aldehyde group because decayed lignin shows no red-purple colour with phloroglucin-hydrochloric acid reagent, which is used for Brauns and Nord's native lignins to detect the conjugated aldehyde group, namely coniferylaldehyde group.

Ultraviolet spectrum of decayed lignin and Nord's native lignin were obtained in the solution of dioxan-water (9 : 1 vol.) by the Beckmann spectrophotometer. Immediately after preparation of decayed lignin, a spectrum of decayed lignin showed the maximum wavelength at 261～262 mμ, shorter than that of native lignin. But, some time after preparation of decayed lignin, the spectrum showed no maximum wavelength. In either case, decayed lignin is greatly different from the native lignin at the view point of ultraviolet spectrum (Fig. 6).

DISCUSSION

Czapek[31] reported that a great amount of "hadromal" was obtained from alcohol or benzene extracts of the wood, which was decayed by the wood-rotting fungus *Merulius lacrymans*, and this alcohol extract gave the very strong red colour with phloroglucine-hydrochloric acid.

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31) F. Czapek, *Ber. Botan. Gese.*, **17**, 166 (1899).

He has also mentioned that "hadromal" was liberated as the result of spliting a bond between lignin and cellulose by an enzyme of "hadromase". According to the present knowlege on the lignin chemistry[32], there is no bond between lignin and cellulose, and rather the ether like bond between lignin and hemicellulose is presumed. Therefore, "hadromal" should be a degradation product from lignin or lignin-hemicellulose complex by an enzyme of "hadromase" with splits on ether linkege.

The acetone extract of decayed wood-meal in author's experiment probably corresponds to Czapek's "hadromal", but the decayed lignin which was prepared from the acetone extract gave no purple red colour with phloroglucin-hydrochloric acid, and rather weak brown colour. These facts may be explained by the different function of the fungi in use, namely that *Merulis lacrymans* in the experiment of Czapek belong to brown rot and *Poria subacida* where as in author's experiment it belongs to white rot.

Higuchi and Kawamura[12] studied on the decay of beech-woodmeal by some white and brown rotting fungi, and found coniferylaldehyde, vanillin and syringaldehyde from ethanol-benzene extract of each decayed wood meal, and suggested that these aromatic aldehydes might arise as the degradation products of lignin.

Henderson[33] investigated on aromatic compounds which was released from birch- and spruce-sawdusts during decomposition by white-rot fungi, *Polystictus versicolor* and *Trametes pini*, finding vanillic acid and vanillin from the ether extract of alkali solution extract of decayed sawdusts. He found also vanillin from the untreated sawdusts. In the case of auther's experiment, no coniferylaldehyde with a trace of vanillin was recognized in the ether extract, and the other various guaiacyl compounds were found in the acetone extract from the decayed wood-meal. In these guaiacyl compounds, guaiacylglycerol-β-coniferylether and vanillic acid-like compound were detected by the reaction of quinonemonochloroimide on the paper-chromatography.

p-Hydroxy-*m*-methoxyphenylpyruvic acid was hardly recognized in the extracts of decayed wood-meal, and was able to be detected in the culture solution, which contained lignin and was cultured at still. This fact is supported by its chemical unstability in the existence of oxygen, that is to say, this compound is easyly oxidised in the porous medium such as wood-meal medium which contained comparably much air, but can accumulate in the still liquid medium which contained little air.

According to author's experiment, coniferylaldehyde and vanillin which were previously mentioned in the report of Higuchi and Kawamura[12], can not be considered as the degradation products of lignin by the function of *Poria subacida.*

The degradation of lignin by the wood-rotting fungus proceeds under acidic condition. Therefore, the occurrence of the degradation of guaiacylglycerol-β-coniferylether is also presumed from the result to studies on the acidic degradation of lignin by Adler[34] and Kratzl[35] as discussed in the previous paper[17]. Recent studies of Freudenberg et al[36,37] suggest that lignin is mainly constituted of the constructional units of guaiacylglycerol-β-coniferylether derivatives.

Considering the results of author's studies and the studies of Freudenberg, Adler and Kratzl on the lignin chemistry, the presumed scheme of degradation of lignin by the wood-rotting fungus *Poria subacida* can be illustrated as shown in Fig. 7.

It may be considered that vanillic acid-like compound was produced as the result of splitting the side chain of guaiacylpropanones by the function of an oxidase of the fungus. Further

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[32] B. O. Lindgren, *Svenk Papp.*, **61**, 669 (1958).

[33] M. E. K. Henderson, *Nature*, **175**, 634 (1955).

[34] E. Adler, J. M. Papper and E. Eriksoo, *Ind. Eng. Chem.*, **40**, 1391 (1957).

[35] K. Kratzl und W. Schweers, *Mh. Chem.*, **85**, 1046 (1954).

[36] K. Freudenberg und A. Sakakihara, *Liebig. Ann. Chem.*, **623**, 129 (1959).

[37] K. Freudenberg und G. Groin, *Chem. Ber.*, **92**, 1355 (1959).

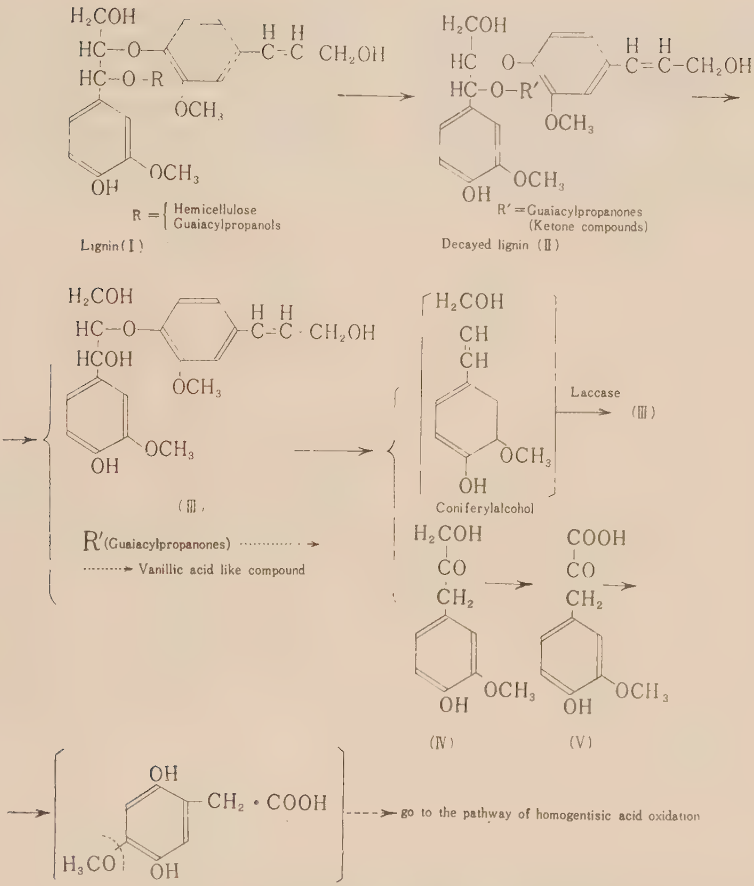

FIG. 7. Presumed Scheme of Enzymatic Degradation of Lignin by *Poria subacida*.

study is now being carried out to confirm the existence of homogentisicase in the medium of *Poria subacida*.

SUMMARY

1. It was confirmed that *p*-hydroxy-*m*-methoxyphenylpyruvic acid occurred in the culture filtrate of *Poria subacida*, in the medium of which native lignin, xylose and asparagine were contained.

2. Guaiacylglycerol-β-coniferylether and vanillic acid-like compound were recognized in the extracts of the decayed spruce wood-meal attacked *Poria subacida*.

3. The decayed lignin extracted from the decayed wood-meal showed the remarkable variation and the existence of the more predominant amount of carbonyl groups, compared with native lignin on their ultraviolet and infrared absorption spectra, and gave no purple-red colour reaction with phloroglucin-hydrochloric acid reagent.

4. Depending upon the above results, the scheme of degradation of coniferous lignin by the function of the wood-rotting fungus *Poria subacida* was presumed and discussed.

Acknowledgements The author expresses his appreciation to Prof. Dr. Takeo Shibamoto and Assist. Prof. Dr. Kyoji Minami, Fac. of Agr., Tokyo University for their guidance and encouragement. He also thanks Prof. Dr. Bunji Maruo, Institute of Applied Microbiology, Tokyo University and Prof. Kei Arima, Fac. of Agr., Tokyo University and Prof. Dr. Shiro Akabori, Fac. of Scinece, Osaka University for their interests and advices, and is grateful to Prof. Dr. Karl Freudenberg, Forshungsinstitut Freudenberg, University of Heidelberg, Germany for giving α-hydroxypropiovanillon and a sample of lignin constituent intermediate.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 739~740, 1960]

Hydrolytic Re-dissociation of Tannin-Protein-Complex by Tannase

Sir:

It is well known that tannin combines with various kinds of proteins including enzyme proteins and forms water-insoluble tannin-protein-complexes (T.P.C.). This fact has so far been applied to purification of some enzymes like barley amylase, etc. The tannin precipitation method, on the one hand, excels other methods by means of salts and organic solvents for precipitating a large quantity of proteins with a small quantity of the reagent; on the other hand, it is comparatively difficult to eliminate only tannin from T.P.C. If an easy removal of tannin were possible, the method would be extensively applied to enzyme purification.

The author now wishes to report the following facts which were found in the studies on the so-called tannase of molds(1). In estimation of tannase activity, turbidity was always brought about when the enzyme solution was added to the reaction mixture containing gallotannin as a substrate. And it gradually disappeared as the reaction proceeded. The more active the enzyme solution was, the more quickly it disappeared. As will be seen in Table I and Figure 1, it seems that it is caused by T. P. C., which is gradually hydrolyzed by tannase.

It was investigated on the additions of tannin

TABLE I. RE-DISSOCIATION OF TANNIN-PROTEIN-COMPLEX.

Used strain			*Pen.* sp No. 80 B'		*Asp. Niger* sp. No. 10	
Bran medium with or without tannin			With	Without	With	Without
Reaction mixture	Extract from above medium		2 (ml)	2 (ml)	2 (ml)	2 (ml)
	2% tannin solution		1	1	1	1
*T.P.C.	Reaction time (hrs.)	0	+++	+++	+++	+++
		24	−	++	−	++
**Ppt. reac.	〃	0	+	+	+	+
		24	−	+	+	+
Reaction mixture	Heat treated above extract		2	2	2	2
	2% tannin solution		1	1	1	1
T.P.C.	Reaction time (hrs.)	0	+++	+++	+++	+++
		24	+++	+++	+++	+++
Ppt. reac.	〃	0	+	+	+	+
		24	+	+	+	+
Reaction mixture	Extract from above medium		1	1	1	1
	malt extract		2	2	2	2
	2% tannin solution		1	1	1	1
T.P.C.	Reaction time (hrs.)	0	+++	+++	+++	+++
		24	−	+++	−	+++
Ppt. reac.	〃	0	+	+	+	+
		24	−	+	−	+

*T.P.C.; Tannin-Protein-Complex, +; turbidity, −; turbidity disappears.
**Ppt. reac.; precipitate by quinine hydrochloride, +; existence of tannin, −. absence of tannin.

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1) H. Nishira and N. Mugibayashi, *Hyôgo Nôka Daigaku Kenkyû Hôkoku*, **1**, 6 (1953), **2**, 1, 5 (1955), **2**, 87 (1956), **3**, 43 (1957), **3**, 119 (1958), **4**, 39 (1959); H. Nishira, *Kagaku*, **29**, 42 (1959).

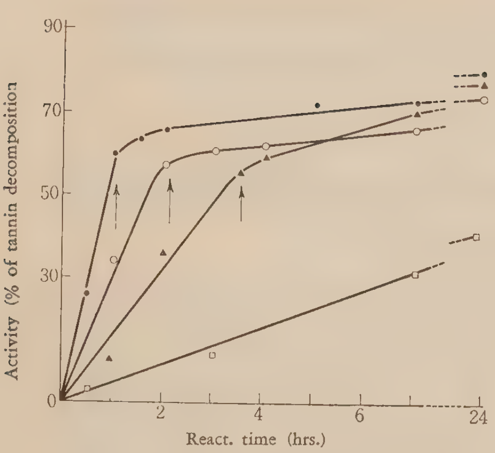

FIG. 1. Recovery of Tannase Activity from Tannin-Protein-Complex.

●—● Original enzyme solution (salting out from 0.2%-tannin-fusuma-koji with $(NH_4)_2SO_4$).
○—○ Enzyme solution from tannin-protein-complex (suspended in water and re-dissociated).
▲—▲ Enzyme solution (obtained from ○—○ by dialysis with tap water for one night).
□—□ Filtrated solution (which was eliminated tannin-protein complex from original enzyme solution).
Arrow-sign in Fig.: disappearing point of precipitate by quinine hydrochloride.

to the enzyme solution extracted from the bran medium, either containing tannin as an inducer[2] or not containing any inducer, T.P.C. was similarly formed, but in the former case T.P.C. disappered after standing at 37°C for 24 hours, and in the latter case T.P.C. almost remained. As shown in Figure 1, the immediate filtrate of the tannin-added enzyme solution had little enzyme activity. On the other hand, the precipitate (T.P.C.) was allowed to suspend in water and was hydrolyzed. That solution had a high enzyme activity. In hydrolysis of the precipitate, the shifting of nitrogen from the precipitate to the solution was ascertained. It was also found by paperchromatography and acid titration that as added tannin was hydrolyzed by the enzyme, the precipitate disappeared.

From the above results it may be concluded that as tannin loses its original protein precipitating ability in hydrolysis by tannase, T.P.C. becomes soluble in water and the protein is recovered. Accordingly, the use of enzymatic removal of tannin by tannase in combination with fractional precipitation by ammonium sulfate makes it possible to recover the enzyme protein from T.P.C. easily. As far as tannase protein[3] is concerned, the above method is very valuable. The application of this method to other enzyme preparations is now under study.

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Received October 8, 1960

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[2] H. Nishira, *Hakkô Kôgaku Zasshi*, **37**, 85, 89 (1959).

[3] H. Nishira and N. Mugibayashi, Annual Meeting of Nippon Nôgei-Kagaku Kai (1960).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 741~742, 1960]

Microbial Formation of Comenic acid from Kojic acid

Sir:

During the course of study on the utilization of kojic acid by microorganisms, it was found by the authors that comenic acid was directly formed from kojic acid by using a bacterium which was isolated from soil.

Since up to now comenic acid has not been known to be directly prepared from kojic acid neither by chemical nor by biochemical methods, consequently little knowledge has been obtained on the oxidation products of kojic acid at the early stage, the formation of comenic acid from kojic acid may be of particular interest on the metabolism of kojic acid.

The bacterium was grown on the bouillon agar medium containing 0.2% of kojic acid at 30°C for a few days. After being harvested and washed, the cells were suspended in distilled water. The cell suspension was added to a reaction mixture contained M/30 phosphate buffer (pH 7.0) and M/20 kojic acid. After the incubation on a shaker at 30°C for 70 hours, comenic acid was isolated from the reaction mixture by the following procedure: the reaction mixture was clarified by centrifuge and was neutrallized. The neutrallized mixture was

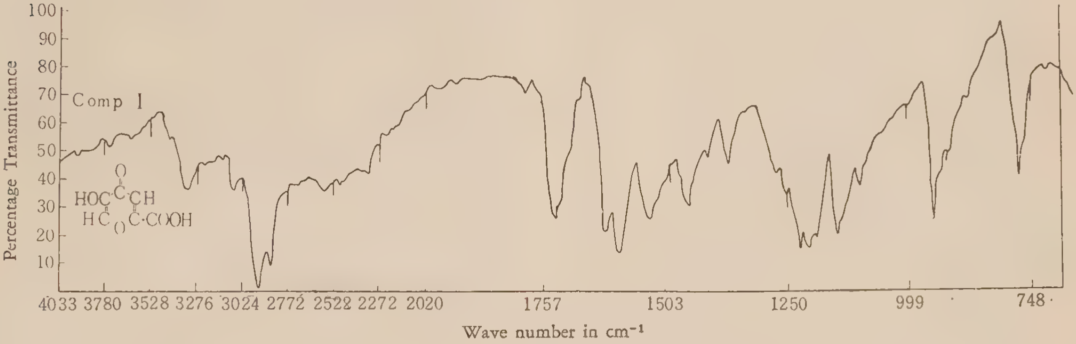

FIG. 1-a. Infrared Absorption Spectrum of (I.)

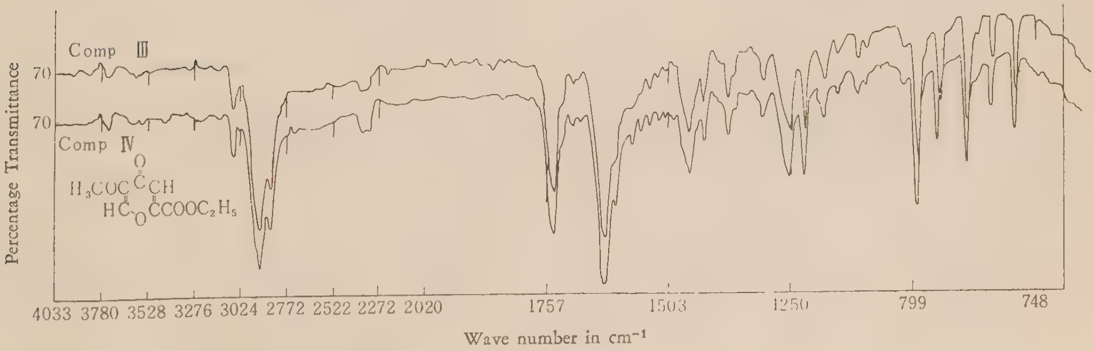

FIG. 1-b. Infrared Absorption Spectra of (III, IV.)

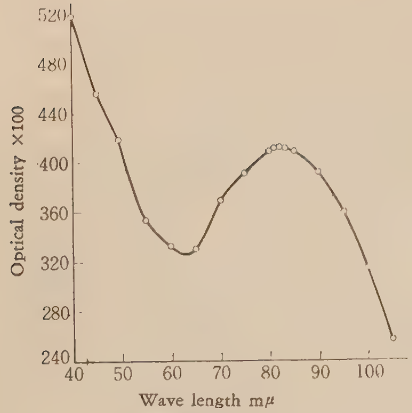

FIG. 2-a. Ultraviolet Absorption Spectrum of (I.)

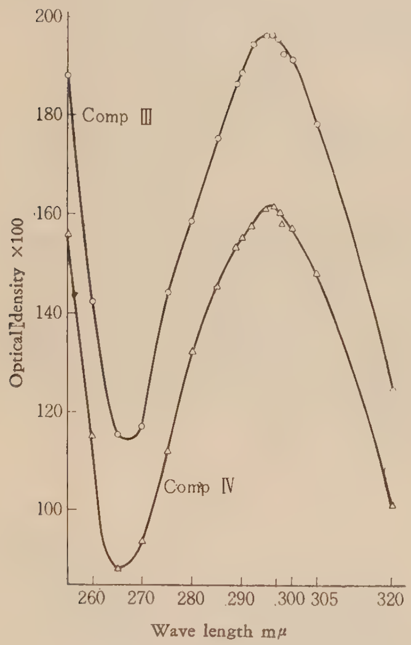

FIG. 2-b. Ultraviolet Absorption Spectra of (III, IV.)

concentrated in vacuo and remaining kojic acid was separated from the mixture by extracting with ether. The mixture was further acidified with sulfuric acid (pH 2) and was carried out the extraction with ether for 80 hours. The ether extract was concentrated in vacuo and the crude crystal obtained was recrystallized from hot water. The crystal (I) was faintly brownish coloured and small plates, which decomposed at 260~261°C. (I) gave a characteristic colour reaction with ferric chloride solution and showed the R_F value of 0.48 on the paper chromatograph with a solvent system consisting of 1 volume of *n*-amylalcohol and 1 volume of 5*n*-formic acid.

Anal. Found: C, 45.75; H, 2.67. Calcd. for $C_6H_4O_5$: C, 46.15:, H, 2.58.

The methyl ether (II) of (I) was prepared by Yabuta's procedure[1], and it was converted to the corresponding ethyl ester (III). (III) melted at 155.5°C and no depression of the melting point was observed when mixed with the authentic ester (IV) which is the ethyl ester of comenic acid methyl ether, synthesized according to Yabuta[2] from kojic acid.

Anal. Found: C, 54.22; H, 5.02. Calcd. for $C_9H_{10}O_5$: C, 54.54; H, 5.02.

The ultraviolet and infrared absorption spectra of the compounds (I, III, IV) are shown in Fig. 1 and 2.

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Received October 18, 1960

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[1] T. Yabuta, *J. Chem. Soc.*, **37**, 1185 (1916).

[2] T. Yabuta and K. Kambe, *J. Agr. Chem. Soc. Japan*, **6**, 516 (1930).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 743~744, 1960]

Biosynthesis of Dipicolinic Acid by a *Penicillium* sp.

Sir:

In the course of study on the biochemical properties of molds isolated from soil, it was found that a considerable amount of dipicolinic acid (DPA) was accumulated in the culture fluid of a mold.

The isolation of DPA from the biological sources was first reported by S. Udo1) from the slime of Natto, a Japanese food prepared from steamed soybeans inoculated with *Bacillus natto*. Recently, many studies have been carried on to find out a possible role of DPA in the bacterial spores, since J. F. Powell2) demonstrated the loss in dry weight of spores of aerobic bacteria during germination was partly ascribed to DPA release to the medium from the germinating spores.

No information, however, has been available with regard to the production of DPA by molds.

The strain, isolated from the soil of Nagano district, Japan, is likely to be *Penicillium citreo-viride*.

The *Penicillium* was incubated stationarily for 7 days at 30°C on the culture media, containing, per l, glucose, 100 g; $NaNO_3$, 1 g; urea, 7 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 1 g and corn steep liquor, 3 ml. After incubation, the clear culture fluid was separated by filtration from the mycelia. As the filtrate was cooled slowly to about 2°C, there resulted many crystals which left white ash on ignition. To remove the possible metal ions contamination from the crystals, the crystals were dissolved in hot water, treated with activated carbon, then hydrogen chloride was added. The solution was cooled very slowly to room temperature, and the resulting crystals were filtered off. Twice recrystallizations of the crystals gave colorless crystalline acid (3.2 g from 670 ml of the culture filtrate) which had no more ash on ignition, and decomposed when melted at 232°C (corr.). The same acid could also be gained from aqueous solution of the colorless crystals through the acid type ion-exchange resins treatment.

The purified crystalline acid was slightly soluble in cold water and was soluble in hot water. On ignition, the crystalline acid decomposed with the marked smell of pyridine. The acid contained carbon and nitrogen, and sulphur and phosphorus were absent. On titration with 0.05 N NaOH, one equivalent of alkali neutralyzed 83.4 g of the acid. *Anal.* Found: C, 50.13; H, 3.03; N, 8.43%. Calcd. for $C_7H_5O_4N$: C, 50.308; H, 3.015; N, 8.382%.

From these data described here, several names of compounds could be picked out from the chemical tables as possible compounds. All of these were pyridine-dicarboxylic acids. Of six possible pyridine-dicarboxylic acids, cinchomeronic acid and dinicotinic acid were first eliminated, because the unknown acid gave strong orange-yellow shade with $FeSO_4$ in aqueous solution, indicating that at least one carboxyl group was in the α position. The dimethyl ester of the acid was prepared, it melted at 121°C, which hinted that the unknown acid was most likely to DPA. DPA was then synthesized from 2,6-lutidine, the dimethyl ester of it was also prepared, the m.p. were 232°C and 121°C respectively. These was no depression of m.p. of admixture with the unknown acid counterparts. In the ultraviolet and infrared absorption spectra of the unknown acid were indistinguishable from these of synthetic DPA.

All these experimental results, mentioned above, indicate this acid from the mold culture filtrate to be DPA.

More detailed results will be published elsewhere.

We are grateful to the Chemical Analysis

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1) S. Udo: *J. Agr. Chem. Soc. Japan*, **12**, 386 (1936).

2) J. F. Powell: *Biochem. J.*, **54**, 210 (1953).

Center of the Government Institute for Industrial Technology of Tokyo for carrying out elementary analysis.

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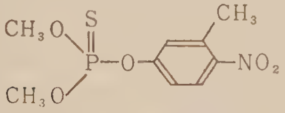
[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 7, p. 744~745, 1960]

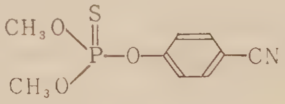
New Low Toxic Organophosphorus Insecticides

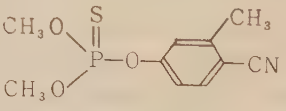
Sir:

As it is well known, phosphorothioates* having 4-nitrophenyl[1] or 4-cyanophenyl[2] groups have high activities towards insects. However, the studies on the correlation between their biological activities and their derivatives have not been reported. To make clear this correlation, the present author prepared a series of derivatives and tested their biological activities.

As the results of these studies, three compounds were found as low toxic insecticides, namely, O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate (A-I), O,O-dimethyl-O-(4-cyanophenyl) phosphorothioate (B-I) and O,O-dimethyl-O-(3-methyl-4-cyanophenyl) phosphoro thioate (C-I).


(A-I)


(B-I)


(C-I)

O,O-Diethyl-derivatives (A-II), (B-II)[2], (C-II), oxo-forms (A-IV), (B-IV), (C-IV) and O-ethylphenyl thiophosphonate-forms (A-III), (B-III), (C-III) had also high insecticidal activities but high toxicities towards mouse (see Table I).

An outstanding characteristic of low toxicities of three compounds (A-I), (B-I) and (C-I) will be apparent from Table I.

It was cleared from the following facts that three compounds occupy an unique position among other isomers, homologues and analogues of three compounds as for the correlation of the toxicities and insecticidal activities. All of other derivatives, namely, 3-methoxy-, 3-chloro-, 3-benzoyloxy-, 2-methyl-, 2-methoxy-, 2-chloro-, 2-benzoyloxy-4-nitrophenyl or 4-cyanophenyl and 3-nitro-4-methyl, 2-nitro-4-methyl-phenyl phosphorothioates etc., showed comparatively low toxicities but lower insecticidal activities than above three compounds.

As a rule, in this series, the correlation of the toxicities and the activities was parallel and introduction of the second substituent to nitrophenyl or cyanophenyl groups reduced their biological activities and the influence of the second substituents on their biological activities were changed according to the substituted

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*Names of phosphorus compounds in this paper were described according to the Drake Committee's Report (Chem. Eng. News, **30**, 4515 (1952).

[1] Jap. Pat. Announced No. 6170 (1956), No. 3449 (1957).

[2] G. Schrader, Die Entwicklung neuer Insektizide auf Grundlage Organischer Fluor- und Phosphor-Verbindungen, S. 59 (1952).

TABLE I. THE BIOLOGICAL ACTIVITIES OF PHOSPHORUS COMPOUNDS.

	Oral toxicity towords mouse LD_{50} mg/kg	Pot-test towards the second generated larvae of rice stem borer (*Chilo suppressalis* WALKER) (spray 4 days later from the encroachment)			
		1/2000	1/4000	1/8000	1/16000
A-I	700～900	100%	100%	100%	100%
A-II	17.5	100%	100%	100%	60%
A-III	30	35.8%	17.0%	18.7%	—
A-IV	20	23.9%	59.1%	—	—
B-I	500	100%	100%	100%	97.2%
B-II	10	100%	100%	100%	—
B-III	40	100%	100%	74.5%	—
B-IV	50	100%	100%	69.1%	—
C-I	500	100%	100%	77.5%	—
C-II	7.5	54.2%	61.7%	85.3%	—
C-III	20	—	—	44%	21.8%
C-IV	25	100%	100%	61.8%	—
Parathion	9.5	98.0	100%	95.6%	—

position.

The abnormal correlations of toxicities and insecticidal activities of (A-I), (B-I) and (C-I) were not found in O,O-diethyl-, oxo-forms or O-alkyl-phenyl thiophosphonate type.

Details will be presented at the next annual meeting of Agr. Chem. Soc. of Japan.

The pot-test was conducted by the following manner. The rice plants, after 45～50 days from planting, were transplanted into a porcelain pot (whose surface area was 1/5000 are) and after elapsing further 60～70 days the plants were applied with the eggs of rice stem borer. Thus, the treated pots were settled in a hatching room. After 4 days from the hatching of the insects, each pot was sprayed with an emulsion of the test compound made by dilution of 50% emulsifiable concentrate composition (comprised of 50 parts active ingredient, 35 parts polyethylene glycol nonylphenylether and 15 parts xylene, by weight) with water. By changing the concentration of the solution to be employed, the Killing % were determined. These tests were repeated five times with each compound and their mean values were showed in Table I.

The author wishes to thank the Sumitomo Chem. Co., Ltd. for permission to publish this work. He is indebted to coworkers in the pesticidal, the biological and the research sections in this laboratory.

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530	left	12	deletopiperazine	diketopiperazine
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Vol. 24, No. 6				
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Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
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Studies on the Adsorption Indicator in the Determination of Sodium Chloride in Soy-Sauce and Miso. Part IV. Determination by the Use of Some Sulfonphthalein Dyes as Adsorption Indicators in Mercurometry. (p. *713～716*)

By Kenji SATO

(*Brewing Experiment Station, Tax Administration Agency*)

(1) Chlorophenol blue (I) and Chlorobromophenol blue (II) are new adsorption indicators for titrating halide ion with $Hg_2(NO_3)_2$. I is suitable for the analysis of Br^-, and II for Cl^- and Br^-. I can be used up to 0.01 N Br^- and II up to 0.01 N Br^-, 0.1 N Cl^- with a color change from yellow to violet, when 0.1 N $Hg_2(NO_3)_2$ solution is employed.

(2) In the volumetric analysis of Cl^- in Soy-Sauce and Miso with mercurometry, as the results of testing of sulfonphthalein indicators (P.R., C.R., T.B., B.P.R., B.T.B., B.C.P., C.P.R., I.P.B., C.P.B., C.B.P.B., B.C.G., T.B.P.B.), bromophenol blue as an adsorption indicator is recommended as the most suitable in the optimum condition. Gelatine, peptone, glucose, furfural etc. do not interfere. And it can be used up to 0.6 N HNO_3 in acid concentrations before titrations.

Studies on the Adsorption Indicator in the Determination of Sodium Chloride in Soy-Sauce and Miso. Part V. Determination by the Use of Diphenylcarbazone as an Indicator in Mercurometry. (p. *716～720*)

By Kenji SATO

(*Brewing Experiment Station, Tax Administration Agency*)

(1) A new adsorption indicator, *p*-dimethylaminobenzylichinerhodanine in acetone solution, is used for the mercurometric titration of halide ion. This indicator is suitable for the analysis of Br^- and the precipitate becomes from red to deep violet at the end point.

(2) A method for the mercurometric determination of Cl^- in Soy-Sauce and Miso is described as more simple and more accurate than the ordinary $AgNO_3$ titration. Diphenylcarbazone (I) is suitable as an indicator and the mechanism of color change is discussed. This method gives satisfactory results in the presence of excess protein, carbohydrate, amino-acids and other organic compounds, but Fe^{3+} should be less than 1.2 mg/ml; the interference of higher Fe^{3+} contents can be avoided by adding of KF or H_3PO_4 solution. The tested solution must be adjusted to pH 1.7～1.1 N HNO_3 at the end point.

I should be added toward the end point and is added preferably before the last 1～2 ml of the titrant. The precipitate is white and turning to blue-violet coloration as the end point is reached. Therefore toe preliaminary titration (35～60°C) should be carried out. Diphenylcarbazide is unsuitable as an indicator for Cl^- analysis.

Secretion of Nucleotides by Yeast Cells. Part IV. Release of Ultra-violet absorbing Materials from the Broken Cells. (p. *721～725*)

By Masataka HIGUCHI and Teijiro UEMURA

(*Department of Agricultural Chemistry, Tohoku University*)

It was examined how the release of U.V. absorbing materials from yeast cells changed, when the cells broken by several treatments were incubated in a citrate or acetate buffer.

In heat shock treatment, the release was stimulated by as much as 30 per cent by the 50°C or 60°C heating for 5 minutes, but almost completely inhibited by the 80°C heating. Considerable increase of the release was also observed, if the cells were treated by means of freezing and thawing or with organic solvents such as acetone, iso-amyl-alcohol and ethyl acetoacetate ester. On sonication the release was stimulated to a certain extent by the short time treatment (30 second), though rather inhibited by the longer one (10 minutes). The stimulative effects on the release by the above treatments were more remarkable in the citrate than the acetate buffer incubation, and these stimulations may be likely due to break the permeabilitic barrier of the cells.

The Simple Micro Method for the Determination of Acrid Substance in WASABI (Japanese Horse Radish) by Paperchromatography. (p. *725～728*)

By Hitoshi YASUDA

(*The Faculty of Liberal Arts and Science, Shinshu University*)

1) About 1 g as exact as possible of WASABI cut nimbly into small pieces is put into the test tube with glass-stopper containing 5 ml of water. Add 1.5 g of NaCl and 5 ml of ether, after incubation for 2 hours at 30°C in the case of total acrid substance, or at once in the case of present substance.

2) After standing for 30 minutes with gentle stirring, ether fraction is poured into another tube containing 2 ml of alcohol and 3 ml of 10% ammoniac solution. After one more extraction in the same way, the water fraction is washed with a little ether. All ether fraction is collected into the first test tube.

3) Ether is evaporated on the water bath at 40°C, and then the test tube is allowed to stand for about 24 hours. After this, the content of it is diluted exactly to 10 ml with water.

4) The test solution, obtained thus, is diluted to various degrees of dilution and 0.01 ml from each of them is spotted on the filter paper for paper chromatography, and eluted with BuOH Ammonia solvent (1 : 1) for 5 cm by ascending method. The spots are developed by spraying ammoniac $AgNO_3$ solution, and the limiting dilution for inspection is decided.

5) The content of acrid substance in WASABI is calculated from the limiting dilution and the limiting sensibility which was decided from the known concentration of allylisothiocyanate in the same condition.

On the Effect of Some Acid Precipitating Agents, upon the Method of Colorimetric Estimation of Glutathione by Saville. (p. *729～731*)

By Masayoshi OGAWA

(*From the Department of Nutrition and Food, Sugiyama Women Senior College Nagoya*)

Saville has shown that S-nitroso derivatives of SH compound can be hydrolysed to the equivalent nitrous acid in the presence of mercuric cations. By adding sulphanilamide and N-1-naphthylethylen diamine, it has been possible to cause this hydrolytically generated nitrous acid to result in the formation of an intensely coloured azo dye, which will be measured colorimetrically against an appropriated blank solution containing water in place of SH solution.

In this report the author describes the application of this method for the determination of tissue SH, extracting it from tissue with acid solution.

For this purpose various acid such as sulfosalicylic acid, oxalic acid, trichloracetic acid, metaphosphoric acid were used to precipitate the proteins of tissue, and prepare a protein-free filtrate, and obtained the following results:

1) The use of metaphosphoric acid does not appear to be satisfactory for the formation of equivalent nitrous acid, in the presence of mercuric ion from S-nitroso derivatives.

2) Sulfosalicylic acid seems to be the best reagent for this purpose.

3) The reaction of zinc with sulforalicylic acid has used to reduce GSSG. Zinc does not interfere the method, and hence GSSG is determined by the difference in the analysis for GSH in sample taken before and after a reduction process.

Studies on Kojic Acid Metabolism by Microorganisms. Part IV. Formation of Kojic Acid from Ethylalcohol by Mold, *Asp. tamarii* var. *crussus*. (p. *732～737*)

By Seiichi NONOMURA, Itsuo ICHIMOTO and Chuji TATSUMI

(*Faculty of Agriculture, University of Osaka Prefecture*)

We studied that kojic acid was formed ethylalcohol by *Asp. tamarii* var. *crussus* with a replacement culture.

In a three or six day culture, in both of liquid culture and calcium carbonate added culture, a few organic acids of the members of TCA cycle were identified using chromatographic technique. Then when these acids (M/250) were added to ethylalcohol medium the yield of kojic acid was increased more than the yield obtained in ethylalcohol medium alone. Monofluoroacetamide, arsenite, diethylmalonate and arsenate inhibited almost completely kojic acid formation. An initial oxidation product of ethylalcohol, i.e., acetaldehyde was trapped by sodium bisulfide and it was identified as 2,4-dinitrophenylhrdrazone.

From these experimental results we propose that kojic acid may give rise to through the following path.

ethylalcohol→acetaldehyde→acetate→TCA cycle——→reversal path of glycolysis——→C_6 compound——→kojic acid.

Enzymatic Studies on the Optical Activity of Amino Acids. Part I. A New Enzymic Resolution of Racemic Glutamic Acid. (p. *737～740*)

By Masao TANAKA, Yuji NAGANO and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.*)

Previously Akita et al. of our laboratory (*Biochemical and Biophysical Research Communications*, **1**, 179 (1959)) reported an enzyme of *Pseudomonas cruciviae* var. *ovalis* which dehydrates specifically L-glutamic acid to L-pyrroglutamic acid, and named it L-glutamic acid dehydrase. A new and simple method of the optical resolution of racemic acid by this enzymic reaction of L-glutamic acid was devised.

Racemic glutamic acid was incubated with a crude enzyme preparation, lyophyled cells of *P. cruciviae*, at pH 8.0, at 50°C, for 4 hrs. L-Glutamic acid was rapidly dehydrated to L-pyrroglutamic acid, while there was apparently no change on D-glutamic acid. L-Pyrroglutamic acid was then separated from D-glutamic acid by using an ion exchange resin. The reaction mixture was percolated through a column packed with sulfonated polystyrene resin (Diaion SK-1, H-cycle) at pH 2.0. The effluent contained only L-pyrroglutamic acid, and D-glutamic acid was selectively absorbed by the ion exchange resin. Absorbed D-glutamic acid was eluated with 2 N ammonia quantitatively. The L-pyrroglutamic acid rich fractions were concentrated and hydrolysed for 2 hrs at 100°C. After filtrating off coagulated proteins, the hydrolyzed concentrate was cooled, and L-glutamic acid monohydrochloride crystallized. L-Glutamic acid was obtained by the recrystallization of L-glutamic acid monohydrochloride at pH 3.2. $[\alpha]_D^{15}$ of this glutamic acid was +31.9 (5%, 10% HCl). The concentration of the eluate yielded D-glutamic acid crystal, $[\alpha]_D^{15}$ of which was found to be −31.1 (5%, 10% HCl).

This new enzymic method with L-glutamic acid dehydrase is thought to be a simpler and more convenient one than those with acylase etc., in order to resolve racemic glutamic acid enzymatically. The enzymic dehydration of L-glutamic acid with a continuous racemization of residual D-glutamic acid, is considered to make possible a complete optical activation of racemic acid.

Enzymatic Studies on the Optical Activity of Amino Acids. Part II. An Optical Activation Method of Racemic Glutamic Acid. (p. *740~745*)

By Masao TANAKA, Yo KATO and Shukuo KINOSHITA

(*Tokyo Research Lab., Kyowa Hakkô Kôgyô Co., Ltd.*)

Previously, a new method capable of optical resolution of racemic glutamic acid with L-glutamic acid dehydrase of *Pseudomonas cruciviae* var. *ovalis* was established, and a posibility of optical activation of racemic glutamic acid was suggested. A method for converting racemic glutamic acid to L-isomer presented in this paper.

As Snell et al. reported, D-glutamic acid was racemized at 50°~100°, at pH 8~10, for 1~4 hrs, with *p*-nitrosalicylaldehyde and Cu^{++} ion, while L-pyrroglutamic acid was not changed in the same conditions. On the other hand, the dehydration of L-glutamic acid with L-glutamic acid dehydrase was active enough in the presence of *p*-nitrosalicylaldehyde and Cu^{++} ion. When racemic glutamic acid was incubated with L-glutamic acid dehydrase, in the presence of *p*-nitrosalicylaldehyde and Cu^{++} ion, L-isomer was dehydrated specifically to L-pyrroglutamic acid, and only residual D-isomer was racemized to racemic glutamic acid, the L-isomer of which was, as soon as formed, dehydrated to L-pyrroglutamic acid again. The repetition of such reactions lead eventually to complete formation of L-pyrroglutamic acid from D-glutamic acid. These both reactions converted racemic glutamic acid to L-pyrroglutamic acid continuously. Formed L-pyrroglutamic acid was easily hydrolyzed with 2 N hydrochloric acid, and L-glutamic acid was obtained.

D-glutamic acid was found in the 4 hrs reaction mixture, but slight loss of glutamic acid was not avoidable because on nonenzymic transamination with *p*-nitrosalicylaldehyde.

It was found that the optimum pH for this activation reaction was 7.5, the optimum temperature was 60~70°C and the optimum mol ratio of glutamic acid, *p*-nitrosalicylaldehyde and Cu^{++}, was 1:0.1:0.05. The yield of L-glutamic acid from racemic glutamic acid was 80~85%, after 4~5 hrs reaction.

Studies on the Foaming Property of the Chicken Egg White. Part I. On the Methods of Measuring Foaminess (I). (p. *746~749*)

By Ryo NAKAMURA and Yasushi SATO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Nagoya*)

In order to fix the method of measuring the foaminess of the chicken egg white, the measuring conditions were studied about a pneumatic method and a whipping method. The measuring conditions that were studied as follows;

(1) measuring temperature, and (2) leaving time of foam about measuring stability for two methods, (3) induced time of gas, (4) the capacity of measuring tube, and (5) sorts of gases for the pneumatic method, (6) the rotatory speed of beater for the whipping method.

With the results of this study, an optimum condition for measuring foaminess could be fixed. The measuring error was within 4~6%.

L-Homoserine Fermentation. Part I. Quantitative Determination of L-Homoserine and Accumulation of L-Homoserine by Microorganisms. (p. *750~754*)

By Hirotoshi SAMEJIMA, Takashi NARA, Chûzo FUJITA and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Hakkô Kôgyo Co., Ltd.*)

(1) Quantitative determinations of L-homoserine in culture broth were made by turbidimetric bioassay with *Escherichia coli* 177~184. The mutant 177~184, which responds either to L-homoserine or to L-threonine plus D,L-methionine, was available for L-homoserine determination, since it failed to respond to D,L-methionine in the absence of L-threonine and since the latter was scarcely present in our assays.

(2) By means of this bioassay, attempts were made to search for microorganisms capable of producing an appreciable amound of L-homoserine, and two strains of threonine-requiring mutants of *Micrococcus glutamicus* (534–Co147 and 534–185) were found to accumulate L-homoserine in their culture fluid remarkably. Approximately 2 mg of L-homoserine per ml. were produced by the mutants, when they were grown on a medium consisting of 10% glucose, 2% $(NH_4)_2SO_4$, 0.2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.03% $MgSO_4 \cdot 7aq$ and 2% $CaCO_3$. The mutants were also found to accumulate about 3 mg/ml of L-lysine on this medium.

(3) Isolation of L-homoserine from the culture liquid was undertaken by the use of ion exchange resins to definitely determine the identity of the amino acid, and found to yield the crude crystals of L-homoserine. The results of paperchromatography, preparation of dinitrophenyl homoserine and bioautography with *E. coli* 177–184, of the L-homoserine preparations indicated that the amino acid produced by the mutants was identical with L-homoserine.

L-Homoserine Fermentation. Part II. L-Homoserine Production in a Natural Medium. (p. *754~759*)

By Hirotoshi SAMEJIMA, Takashi NARA, Chûzo FUJITA and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Hakkô Kôgyo Co., Ltd.*)

(1) A natural medium for L-homoserine production with *Micrococcus glutamicus* must be supplied with natural L-threonine and biotin sources, because of the organism being a L-threonine and biotin requiring mutant. The L-homoserine producing ability of the organism was found to vary more greatly depending on the kind and amounts of natural L-threonine sources than those of biotin sources. The most complete medium for such natural materials consisted of 10% glucose, 2% $(NH_4)_2SO_4$, 0.5% yeast extract, 0.1% K_2HPO_4, 0.03% $MgSO_4 \cdot 7aq$ and 2% $CaCO_3$, supplied with either 1% NZ-amine or 1.5% peptone. The yields were approximately 7 mg/ml of L-homoserine.

(2) Addition of ammonium sulfate and ammonium chloride as inorganic nitrogen sources was found to give better yields of L-homoserine than other sources.

(3) Optimal temperature, aeration, and initial pH for L-homoserine production were definitely determined.

(4) No significant improvement of yields was made in L-homoserine production by adding L-aspartic acid intermittently to the above medium, whereas feeding of fumaric acid gave a slight increase of L-homoserine production.

(5) The replacement of glucose by sucrose or maltose as a carbohydrate source was noted to produce L-homoserine as well as glucose. In particular, the maltose medium yielded more L-homoserine and less alanine and valine than media in which other carbohydrates were employed as sugar sources. Only about 3 mg/ml of L-homoserine was produced on molasses media.

Studies on the Oxidation of Steroids by Microorganisms. Part I. On the Oxidation of Reichstein's Substance S by *Gloeosporium* and *Glomerella* Species. (p. *759~762*)

By Eiji KONDO and Eitaro MASUO

(*Research Laboratory of Shionogi and Co., Ltd.*)

We have investigated on the microbiological oxidation of Reichstein's substance S by two species of Genus *Glomerella* and three species of Genus *Gloeosporium* and it has become apparent that these strains—plant pathogenic fungus—have the powerful oxidizing activity except one, *Gloeosporium kaki*. The following results were obtained.

(1) With the use of *Glomerella mume*, *Glomerella cingulata* and *Gloeosporium foliicolum* 11α-hydroxylation of substrate have occurred and 11-epihydrocortisone was obtained in good yield.

(2) On the other hand 1-dehydrogenation of substance S was observed with the use of *Gloeosporium olivarum*.

(3) *Gloeosporium olivarum* is classified into Genus *Glomerella* in the perfect stage, but the oxidation pattern between *Glomerella* species and *Gloeosporium olivarum* applied in this study was greatly different.

Studies on the Oxidation of Steroids by Microorganisms. Part II. On the Oxidation of Reichstein's Substance S by *Helminthosporium* Species. (p. *762~766*)

By Eiji KONDO

(*Research Laboratory of Shionogi and Co., Ltd.*)

29 species of Genus *Helminthosporium* were used to investigate their oxidation products from Reichstein's substance S. The results were as follows:

(1) 1-Dehydrogenation of substance S was observed in few strains and with the use of *H. zizaniae* and *H.*

gramineum Δ^1-substance S was obtained in good yield.

(2) Hydroxylated products were also found with the use of other strains and these obtained compounds were described below.

Hydrocortisone [*H. sigmoideum* (H-40)]
11-Epihydrocortisone [*H. sigmoideum* (H-37, H-39)]
6β-Hydroxy-substance S [*H. leersii* (H-20)]
14α-Hydroxy-substance S [*H. avenae* (H-9)]
2β-Hydroxy-substance S [*H. tritici-vulgaris* (H-25)]

(3) On the other hand reduced products of substance S such as allopregnane derivative and 20-hydroxy-compound were detected under the aerobic condition, but these results will be described later.

Studies on the Hardwood Dissolving Sulphate Pulp. Part I. Behavior of Hardwood during Prehydrolysis Sulphate Cooking. (1) The Effect of Prehydrolysis upon Pulp Properties. (p. *766∼772*)

By Shonosuke TAKAHASHI, Katsumi NISHIYAMA and Yusaku FUKUDA

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The effect of prehydrolysis followed by proper sulphate cooking on the analytical properties of resulted pulp was investigated using beech as raw material. Most of pentosan in the wood proved to be readily removed by this process and the chemical purity of the resulted pulp was easily elevated to such a grade as suitable for chemical conversion in viscose process. Small amount of residual pentosan, two to three % based on unbleached pulp, however, was found to be extremely resistant to the action during the prehydrolysis sulphate process. When a violent condition of prehydrolysis was used to remove this portion of pentosan, serious degradation of cellulose took place. Hence the process may not be considered as suitable for the preparation of acetate grade pulp in which particularly low content of pentosan is required.

As the condition of prehydrolysis was intensified, the remained lignin in unbleached pulp after alkaline cooking was somewhat reduced. The tendency that the bleachability of the pulp become poor as usual observed in the softwood pulping was not recognized. Some other aspects concerning the relationship between prehydrolysis and pulp properties were also investigated and discussed.

Studies on the Hardwood Dissolving Sulphate Pulp. Part II. Behavior of Hardwood during Prehydrolysis Sulphate Cooking. (2) Change of Analytical Properties of Unbleached Pulp in the Course of Sulphate Cooking. (p. *772∼776*)

By Shonosuke TAKAHASHI and Yusaku FUKUDA

(*Research Institute of Nippon Pulp Industry Co., Ltd.*)

To follow the previous investigation, delignification and depolymerization during sulphate cooking of prehydrolysed hardwood chips were investigated and following results were obtained.

Delignification in the sulphate cooking stage was accelerated as the preceding prehydrolysis was prolonged, and there was no evidence of the deactivation of lignin which has been observed when softwood was subjected to the prolonged prehydrolysis beyond some limitation.

It was found that the depolymerization in alkaline cooking has a favorable effect on the analytical properties of the resulted pulp within a certain limit. It is possible to reduce the amount of pentosan as well as beta cellulose fraction remained in the resulted pulp by prolonging the duration at the sulphate cooking stage. Contrarily the depolymerization in the acid prehydrolysis tends to accumulate the degrated cellulose fractions in the pulp. This fact seems to show that the mechanism of depolymerization at the sulphate cooking stage is substantially different from that in the prehydrolysis stage.

Studies on the Bacteriophage and the Desoxyribonuclease of a *Bacillus subtilis* strain Producing Amylase. Part III. General Enzymatic Properties of Desoxyribonuclease and the Influence of Various Reagents. (p. *777∼782*)

By K. WATANABE and K. YAMAFUJI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu Uuiversity*)

This paper deals with the DNase of the supernatant fluid of sonorated *Bacillus subtilis* var. amyloliquefaciens Fukumoto-K59 which is a phage sensitive bacterium and produces amylase to its culture medium. Viscosimetric determination was used for the most parts to measure this DNase activity and ultra violet absorption ($260 m\mu$) method was employed complementary.

The enzyme was activated by calcium ion in addition to magnesium and manganese ions, and inhibited by both ethylenediaminetetraacetate and sodium citrate. Moreover since the optimum pH is about 8.5 (previous report), it may be naturally concluded that this DNase belongs to the group of DNase I. pH Stability was recognized in the range of 5.5∼7.5 and at pH 8.5 this enzyme gradually lost its activity. Optimum temperature for the activity was 32∼35°C. The action of this enzyme was prevented by sulfhydryl-reagents such as *p*-chloromercuribenzoate, potassium ferricyanide and monoiodoacetate, but some other substances, e.g. cyanide, azide, oxine, cysteine, nitroso-R-salt did not remarkably affect the enzyme reaction. Some cations, cupric, mercuric and zinc ions were found to cause inhibition the enzyme. On the contrary, stannous ion seemed to be somewhat accelerative toward this enzyme. The effect of purine derivatives and commercial carcinostatic agents (8-azaguanine, 8-azaxanthine and 6-mercaptopurine) were examined, and the majority of these drugs had no significant influence on this DNase activity, but 6-mercaptopurine was inhibitive at the concentration of 10^{-3} M.

Studies on the Synthesis of L-Amino Acids. Part I. A Synthesis of L-Proline from L-Glutamic Acid. (1) (p. *782~785*)

By Masao TANAKA, Teruo KISHI and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Hakkô Kôgyo Co., Ltd.*)

As an investigation of the synthesis of L-amino acids, a synthesis of L-proline from L-glutamic acid was studied. The results are as follows:

(1) By treatment of γ-esters of L-glutamic acid with sodium borohydride in water solution, the esters were reduced and cyclized to L-proline without any change of the configuration.

(2) The following results on the conditions of this reaction, were obtained. γ-Methyl ester and γ-ethyl ester had the same reactivity, water was the best reaction medium because it dissolved the ester well, and the better results were obtained at the lower temperature. Although the higher concentration of the ester gave the more formation of L-proline, the best yield of L-proline was given by the concentration of about 200 mg/ml of the ester because of its solubility in water. The molar ratio of the ester to sodiumborohydride of 1 : 1.4 was found to give the best yield. The reduction of the ester with lithiumborohydride or calciumborohydride produced by the addition of lithiumchloride or calciumchloride to the reaction solution could not give rise to better results.

Although the best yield of L-proline was 34% of theoretical yield, no product was present other than L-proline and L-glutamic acid, and the amount of the recovered L-glutamic acid after the hydrolysis of the reaction mixture was about the same as the ester which did not change to L-proline. Based on the result that the increased basicity of the reaction mixture gave lower yield of L-ploline, about 34% of the low yield of L-proline is considered to be ascribed to hydrolysis of the ester.

(3) The mechanism of the reaction is presumed to be one of the following three:

i) The reduction of 2-pyrrolidone-5-carboxylic acid given by the cyclization of γ-ester of glutamic acid.

ii) The reduction of Δ'-pyrroline-5-carboxylic acid, the cyclization from of L-glutamic acid semialdehyde given by the reduction of the ester.

iii) The cyclization of α-amino-δ-hydroxyvaleric acid given by the reduction of the ester.

Studies on Thiamine of Cereals and Beans by the Photographic Method. Part VII. The Distribution of Thiamine in the Seed of Soybean. (p. *786~788*)

By Shinjiro CHIKUBU, Shoji YANAI and Tatsuo TANI

(*Food Research Institute, Ministry of Agriculture and Forestry*)

The distribution of thiamine in the seed of soybean has been studied by the photographic method of thiochrome fluorescence and chemical assays of thiamine have been carried out on three fractions of the seed (seed coat, cotyledon and embryo).

According to the photographic observation, there has been highest concentration of thiamine in the cotyledon, rather higher in the embryo and low in the seed coat. The observation of the cotyledon in details has indicated that the thiamine had appeared to be higher concentrated in the cotyledon epidermis, particularly in the aleurone cells of cotyledon. In embryo, it has not been recognized to be concentrated mainly in the procambium and meristem.

The chemical assays have given almost same results as the photographic observation of the thiochrome fluorescence.

Studies on the Production of D-Araboascorbic Acid by Penicillium. Part I. Separation and Identification of D-Araboascorbic Acid. (p. *788~791*)

By Takeshi TAKAHASHI, Moritaka MITSUMOTO and Hisashi KAYAMORI

(*Department of Agricultural Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo*)

This is the first report on the production of D-araboascorbic acid by a mold. It was very rare case that D-araboascorbic acid has been found in biological materials or products.

The strain belongs to the Penicillium isolated from the soil produced D-araboascorbic acid from glucose or sucrose.

After incubating for 5~7 days in 25°C stationarilly, the reducing substance was extracted from the broth by the method using the anion exchange resin and white crystals which have strong reducing power to 2,6-dichlorophenol indophenol were obtained.

From the experiments on the melting points, chemical analysis, rotation, ultra violet and infra red spectra on the free crystal and 2,4-dinitrophenyl hydrazone, it was proved that the reducing substance was D-araboascorbic acid.

Studies on the Fermented Milk. Part VII. Studies on the Non-Fermentable Milk (2). Effect of Small Amount of Penicillin in Milk on the Lactic Acid Fermentation by *L. bulgaricus* and Heat-stability of Penicillin in Milk. (p. *792～794*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co., Ltd.*)

Different concns. of penicillin (I) were added to skim milk, heated to 100°C for 30 min. inoculated with 3% starter culture of *Lactobacillus bulgaricus*, incubated at 37°C and the acid production is determined by titration.

The followings became apparent about the effect of (I) in milk on the lactic acid fermentation by *L. bulgaricus*:

(1) 1.0 I.U./ml. of I in milk before pasteurization inhibit completely the acid production.

(2) Acid production is partially inhibited at the lower concns. of I than 1.0 I.U./ml and does not decrease at the 1/100 I.U./ml of I in milk.

Moreover, on the heat-stability of I in milk the followings became clear:

(1) I in water soln. is completely destroyed by heating 100°C for 30～45 min.

(2) 1/30～2.0 I.U./ml of I in milk is not inactivated by heating 100°C for 30～45 min. Heating 100°C for 120 min. inactivate 1/30 I.U./ml of I in milk but does not higher concns. of I.

(3) Lower concns. of I than 1/10 I.U./ml are inactivated by 120°C 60 min. but 1.0 I.U./ml still decrease the acid production.

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Polarographic Studies on Storage of Meats. Part X. Influence of Gamma-Ray Irradiation on Protein Wave of Beef. (p. *795~800*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

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In the previous report we have examined the influence of the γ-ray irradition on the change in the component of the raw beef which was vacuum-packed into polyethylene-cellophane bag. The result of the above experiment has shown that in the case of the irradiated beef after the 20°C storage, there is a characteristic change in each component content with increasing days of 20°C storage and dosages of the γ-ray.

In the present paper, we have carried out the experiment on the change in the shape, height, and crossing point of the protein wave of the irradiated beef during 20°C storage under the same conditions as in the previous experiment.

The results of the experiment mentioned above are as follows:

(1) When the protein waves of the water and the 7% NaCl extracts from the irradiated beef are estimated immediately after irradiation, a prominent change in the shape, the height, and the crossing point of their protein waves, is not seen.

(2) In the case of the protein wave of the water extract from the irradiated beef after the 20°C storage, there is a characteristic change in the spape of the protein wave with increasing days of 20°C storage and dosages of the γ-ray.

(3) In the case of the protein wave of the water extract from the irradiated beef after 20°C storage, there is an increase in the height of the second wave than that of the first wave with increasing days of 20°C storage. Consequently, each crossing point from the polarograms in various concentrations of NH_3 is not seen. But in the case of dosages more than $200\times10^4\gamma$, their crossing points are seen.

(4) In the case of the protein wave of the 7% NaCl extract from the irradiated beef after ihe 20°C storage, a remarkable change in the shape is not seen. But there is a change in the wave-height with increasing days of 20°C storage and dosages of the γ-ray.

(5) In the case of the protein wave of the 7% NaCl extract from the irradiated beef after 20°C storage, a remarkable change in the shape of polarograms in various NH_3-concentrations, is not seen. With increasing dosages of the γ-ray, there is an increase in the height of the crossing points obtained. But in the case of dosages more than $200\times10^4\gamma$, there is a decrease in the height of those up to storage period of 40 days.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part VI. The influence of Tocopherol, Carotenoid and Sterol isolated from Soybean Oil on the Color Reversion of Refined Soybean Oil. (p. *800~807*)

By Ichiro HARADA, Yutaka SARATANI and Masao ISHIKAWA

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Unsaponifiable matters such as tocopherol, carotenoid and sitosterol were isolated from soybean oil. The influence of them on the color reversion of oxidizing soybaen oil were studied by adding them into soybean oil substrate which had been removed unsaponifiable matters.

Obvious browning was observed only with tocopherol. Browning caused by tocopherol was accelerated with addition of metals. Their order of activity were $Cu^{\cdot\cdot} > Na^{\cdot} > Fe^{\cdot\cdot\cdot} > Fe^{\cdot\cdot} \geqq Cr^{\cdot\cdot\cdot} > Al^{\cdot\cdot\cdot} > Ni^{\cdot\cdot} > Co^{\cdot\cdot}$, and chloride showed more powerful pro-oxidant action than sulfate.

The color density of oxidized oil increased according to the quantity of tocopherol and iron ions added, however, iron gave more marked effect at the lower concentration.

Evidence was obtained to indicate that heavy metals added complexed with fat hydroperoxides, and these complexes formed themselves into cloudy precipitates in the oil at high peroxide level.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part VII. Formation of Quinoid Compounds from Natural Tocopherol in Soybean Oil. (p. *807~811*)

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On the basis of spectrophotometric data of oxidizing soybean oil and its unsaponifiable matters, it has been shown that the brown color which develops during aeration of oil results from conversion of natural tocopherol to tocopherol-O-quinones.

However, in the absorption spectrum of the oil oxidized without aeration, formation of O-quinone has not been confirmed, and it has been shown from the spectral absorption in the ultra violet region that a considerable amount of tocopherol remained undecomposed in the oil.

From the above observations and previous results, it has been concluded that the color reversion of refined soybean oil is due to the conversion of natural tocopherol to quinones, but various results on color development are obtained according to the conditions of oxidation of oil.

Studies on Pungent Principles of Red Pepper. Part VI. On Chemical Constitutions of the Pungent Principles (3). Hydrogenation Products, Bromide and Decomposition Products. (p. *811~814*)

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The writers reported already that two pungent principles, I and II, were in red pepper. In the present paper, presumptions on chemical constitutions of the pungent principles were reported.

I. m.p. 65.9~66.3°C. Its elementary composition accorded with $C_{18}H_{27}O_3N$. Its 1 mole absorbed 1 molar proportions of hydrogen in presence of palladium. I gave dibromide (m.p. 112~113.5°C). Its ultra-violet and infra-red spectra showed the same band as Crombie's N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-trans-6-enamide. Its decomposition with hydrochloric acid gave vanillylamine.

Accordingly, the pungent principle I was presumed to be N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-trans-6-enamide.

II. Its melting point was 65.6~65.8°C and undepressed on admixture with hydrogenated I. Its elementary composition accorded with $C_{18}H_{29}O_3N$. II gave no bromide having a constant melting point. Its infra-red spectrum showed no band at $968\,\mathrm{cm}^{-1}$, but it showed the same band as hydrogenated I. II gave the same R_F on many paper chromatographies varying developing agents as hydrogenated I. Its decomposition with hydrochloric acid gave vanillylamine.

Accordingly, the pungent principle II was pressumed to be saturated I, viz. N-(4-hydroxy-3-methoxybenzyl)-8-methylnonanamide.

Studies on Transamination by Microorganisms. Part I. The Distribution of Transamination in Bacteria. (p. *814~819*)

By Hideo KATAGIRI, Kenji SODA and Tatsurokuro TOCHIKURA

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(1) The distribution of transaminases revealing the reactions between α-keto acids such as α-ketoglutarate, pyruvate, phenylpyruvate and oxaloacetate, and various L-amino acids were observed with 15 strains of various kinds of bacteria by a modified method of determination of amino acids using circular paper chromatography.

(2) Aspartate and phenylalanine were generally found to be active donors of amino group to α-ketoglutarate with all the bacteria employed.

(3) Leucine, isoleucine, valine, norvaline, lysine and phenylalanine were highly active donors of amino group to α-ketoglutarate in *Flavobacterium fuscum*, *Flavobacterium flavescens*, *Achromobacter liquidum* and *Xanthomonas citri*.

In *Escherichia coli* 2b and *Alcaligenes faecalis*, aspartate acted as more active donor than in the above-mentioned strains and lysine-α-ketoglutarate transamination was not observed.

(4) Aspartate and ω-amino acids such as β-alanine, γ-aminobutyrate and ornithine revealed powerful transamination to α-ketoglutarate with *Achromobacter superficialis* and *Achromobacter polymorph*.

(5) Not only aspartate, phenylalanine, ornithine and tryptophan, but also α-aminobutyrate and α-alanine showed higher transamination activity in the case of *Bacillus natto* SN, *Bacillus roseus*, *Proteus vulgaris* and *Corynebacterium sepedonicum*.

In addition to these amino acids, leucine, isoleucine, valine and norvaline were highly active donors to α-ketoglutarate in *Pseudomonas fluorescens* and *Bacillus thiaminolyticus*.

(6) Activity of transamination of L-amino acids to pyruvate, phenylpyruvate or oxaloacetate was generally lower than that observed in the case of α-ketoglutarate excepting high activities of transamination of leucine or norvaline to phenylpyruvate with *Agrobacterium tumefaciens*, of α-aminobutyrate to pyruvate with *Proteus vulgaris* and with three strains of *Bacilli* and of β-alanine or γ-aminobutyrate to pyruvate with *Pseudomonas fluorescens*.

Studies on Transamination by Microorganisms. Part II. On the Fractionation of Bacterial Transaminases and on their Properties. (p. *819~824*)

By Hideo KATAGIRI, Kenji SODA and Tatsurokuro

TOCHIKURA

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(1) The fractionation of transaminases in cell free extracts of *Flavobacterium fuscum* and of *Achromobacter superficialis* was carried out.

Some properties of the enzyme preparations were investigated and compared the specificities of their actions in order to approach the problem of the multiplicity of transaminases.

(2) The transaminases of *Flavobacterium fuscum* were partially fractionated and the influence of heat treatment, inhibitors and pH on the activities was investigated. And the transaminations relating to aspartate-, leucine-, lysine- and phenylalanine- α-ketoglutarate systems were found to be catalyzed by different enzymes.

(3) Transaminases of *Achromobacter superficialis* were fractionated into two groups, i.e. γ-aminobutyrate- or aspartate-system and β-alanine- or ornithine-system.

(4) From the different affinity of the coenzyme, γ-aminobutyrate- or aspartate-system was further divided into γ-aminobutyrate-α-ketoglutarate and aspartate-α-ketoglutarate transaminases.

Moreover, β-alanine- or ornithine-system was again divided into two kinds of enzymes according to their behavior to inhibitors, pH and incubation temperature.

L-Homoserine Fermentation. Part III. L-Homoserine Production in Chemically Defined Media.

(p. *824～828*)

By Hirotoshi SAMEJIMA, Takashi NARA, Chūzo FUJITA and Shukuo KINOSHITA

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(2) In attempts to devise a chemically defined medium for L-homoserine production, biotin and L-threonine were added in various amounts to a basal medium consisting of 10% glucose, 2% $(NH_4)_2SO_4$, 2% $CaCO_3$, 0.1% K_2HPO_4 and 0.03% $MgSO_4$ 7aq. The results showed that 13 to 15 mg/ml of L-homeserine and 9 mg/ml of L-lysine were produced in the culture fluid when over 5 γ/l of biotin and 400 to 500 γ/ml of L-threonine added to the basal medium. It was also found that the conversion of L-lysine to L-homoserine fermentation took place when the addition of L-threonine was increased to more than 300 γ/ml, and that the addition of less than 5 γ/l of biotin gave rise to production of L-glutamic acid.

(2) The effects of carbohydrate sources, inorganic nitrogen compounds, trace elements, vitamins and organic acids on L-homoserine and L-lysine production were investigated in the above synthetic medium.

It is apparent from the results obtained that L-homoserine production tended to be made more unstable by the addition of some of these compounds than L-lysine production.

L-Homoserine Fermentation. Part IV. Effect of D,L-Methionine and L-Threonine on L-Homoserine Fermentation. (p. *828～832*)

By Hirotoshi SAMEJIMA, Takashi NARA, Chūzo FUJITA and Shukuo KINOSHITA

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(1) From experiments to investigate the effects of amino acids on L-homoserine production, D,L-methionine was demonstrated to bring about a marked reduction of L-homoserine formation, whereas L-lysine production was considerably stimulated by D,L-methionine. It was also clarified that L-crystine and D,L-homocysteine exerted a slight inhibitory effect on L-homoserine production.

(2) Further results on the inhibitory effect of D,L-methionine on L-homoserine production are as follows:

1) The reduction of L-homoserine yields and the stimulation of L-lysine yields were evidently caused by increasing the addition of D,L-methionine eventually up to over 200 γ/ml. In such cases, it was also shown that the total yields of L-homoserine and L-lysine were always some 20 to 25 mg/ml, independent of the amounts of D,L-methionine added.

2) Intact cells harvested from a synthetic medium plus D,L-methionine were able to synthesize less L-homoserine and more L-lysine than those from the synthetic medium alone.

3) D,L-methionine was found to exert no inhibitory effect on the activity of enzymes to synthesize L-homoserine.

These results led to a conclusion that D,L-methionine inhibits the formation of enzymes responsible for L-homoserine synthesis.

(3) L-Threonine was also demonstrated to inhibet enzyme activities concerned with L-homoserine and L-lysine synthesis.

(4) On the assumption that a common precursor for L-homoserine and L-lysine synthesis from L-aspartic acid is designated compound X, it was presumed from the above feed back mechanisms that the sites, of enzyme repression by D,L-methionine, and of enzyme inhibition by L-threonine, might be between compound X and L-homoserine and before compound X, respectively.

L-Alanine Fermentation. Part I. L-Alanine Fermentation by No. 483 strain. (p. *832～837*)

By Hirotoshi SAMEJIMA, Takashi NARA, Chūzō FUJITA and Shukuo KINOSHITA

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Fermentative production of alanine by a bacterial strain (No. 483 strain) which was a Gram-negative, motile, non-sporulating and short rod, was investigated.

A significant amount of alanine was accumulated extracellularly when this organism was grown in a medium containing 10% glucose, 2% ammonium sulfate, 0.2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, and 2% $CaCo_3$. After isolation and purification of alanine from the fermented broth, the product was identified to be L-form.

Addition of ammonium sulfate or urea alone during the fermentation did not show any increase of alanine yield. However, marked increase of alanine production was observed when either pyruvic acid or lactic acid was fed with ammonium source. This phenomenon also took place even under subsequent unaerated condition after the addition of ammonium lactate. This might suggest the presence of a coupled reaction of alanine dehydrogenase and lactic dehydrogenase in this organism.

It was also found that this strain needed a certain component in yeast extract for its growth and alanine production.

L-Alanine Fermentation. Part II. Enzymatic Aspects on L-alanine Fermentation by No. 483 Strain. (p. *838～844*)

By Hirotoshi SAMEJIMA, Chūzō FUJITA, Takashi NARA and Shukuo KINOSHITA

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Alanine production by a bacterial strain (No. 483 strain) was investigated from a enzymatic view point.

In experiments using cell free extract of the organism, potent activity of DPN-dependent L-alanine dehydrogenase was observed. This enzyme showed pH optimum for oxidative deamination of alanine at 9.8. The activity of this enzyme was markedly inhibited by higher concentrations of the substrate, alanine. On the contrary, alanine formation by transaminase reaction was almost negative in this organism.

As DPN-dependent lactic dehydrogenase activity was not found under experimental conditions used, the possibility of the presence of a coupled reaction between alanine dehydrogenase and lactic dehydrogenase might be very low. This suggested that oxidation of lactatate to pyruvate in this organism might be catalized by an enzyme system which needed some hydrogen acceptor other than DPN.

Transamination reaction between α-ketoglutarate and alanin was also negative even though rather strong transaminase activities were found between α-ketoglutarate and aspartate, phenylananine or valine. From this evidence, it seemed that alanine was a sort of end product in this organism.

Addition of 10^{-5} to 10^{-3} M of 2,4-D to the culture stimulated the velocity of alanine formation at an early stage of the fermentation, while the lower concentration (10^{-6} M) of 2,4-D caused strong inhibition of alanine production.

The mechanism of alanine formation by this strain was discussed from the above mentioned results.

Studies on the Oxidation of Steroids by Microorganisms. Part III. On the Oxidation of Reichetein's Substance S by *Cercospora* Species. (p. *844～847*)

By Eiji KONDO, Kazuyuki MORIHARA, Yoshio NOZAKI and Eitaro MASUO

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Microbiological oxidation of Reichstein's sustance S was carried out with the use of 34 species of Genus *Cercospora*.

In many species among these microorganisms tested the ability of introduction of oxygen into position 11α of the steroid nucleus was recognized and the use of *Cercospora melongenae* is most favourable to obtain 11-epihydrocortisone in excellent yieldr

When *Cercospora zinniae* was used we have found that this strain has the ability of producing hydrocortisone and 11-epihydrocortisone and that the yields of these two compounds can be varied to some extent by the methods of hydroxylation.

Studies on the Oxidation of Steroides by Microorganisms. Part IV. Introduction of Double Bond into Ring A. (p. *847～852*)

By Eiji KONDO and Eitaro MASUO

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Four strains of Fungi Imperfecti—*Gloeosporium olivarum*, *Helminthosporium zizaniae*, *Helminthosporium gramineum*, *Graphiola cylindrica*—were used to investigate the oxidation activity of various steroid sustrates such as progesterone, Reichstein's substance S, cortisone and hydrocortisone.

We have found the ability of 1-dehydrogenation of the steroid nucleus with each strain, but the stereochemical specificity of substrates has also been observed.

The obtained results were as follows:

(1) *Gloeosporium olivarum* could dehydrogenate progesterone, substance S and Δ^1-derivative prespectively could be obtained in excellent yield. When cortisone was used as the steroid substrate in the oxidation of this fungus, most of the transformation product obtained was Δ^1-Reichstein's substance U and a minor amount of prednisone and Reichstein's substance U were isolated.

(2) With the use of *Helminthosporium* sp. we have obtained Δ^1-substance S from substance S in good yield, but these fungus could not dehydrogenate hydrocortisone.

(3) *Graphiola cylindrica* could poorly oxidize progesterone, substance S and cortisone. But when dydrocortisone was used as the steroid substrate it was converted predominantly to prednisolone.

Enzymatic Studies on the Optical Activity of Amino Acids. Part III. Racemization of Glutamic acid by bacteria. (p. *852～856*)

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For the purpose of obtaining the organisms which reacmize glutamic acid, 79 strains of bacteria belonging to genus *Bacillus* were tested for ability to grow on a medium which contained D-glutamic acid as a sole source of nitrogen. Twenty eight *Bacilli* were found to grow on this medium, although the majority of the strains did not show to heavy, growth as in a L-glutamic acid medium.

An examination of the formation of L-glutamic acid from D-glutamic acid with acetone dried cells of these 28 strains showed that some of them including one strain of *Bacillus licheni*, possesed an activity of converting D-glutamic acid to the L-isomer. Some observations were made on the mechanisms of conversion of L-glutamic acid to the L-isomer by *B. licheni*. The results of these studies are presented.

Acetone dried cells and Sonic extracts were active with D-glutamic acid in the conversion, while the activity with the L-isomer was usually much more weak than that with the D-isomer. The rate of conversion of D-glutamic acid to the L-isomer was increased by the addition of pyruvate, and this stimulation by pyruvate was more enhanced in dialyzed sonic extracts than in acetone dried cells. Experiments on the synthesis of glutamic acid from α-ketoglutaric acid and two isomers of alanine showed that the glutamic acid produced from α-ketoglutaric acid and either L- or D-alanine was the D-isomer, and the L-isomer was not synthesized. The rate of alanine formation from pyruvic acid and D-glutamic acid by a transamination was more rapid than from pyruvic acid and L-glutamic acid. On the basis of these observations, it was assumed that the enzyme preparation of *B. licheni* contained a very active alanine racemase and a relatively active D-transaminase, while L-transaminase of this preparation was weak. Then, the observed conversion of D-glutamic acid to the L-isomer could occur by the combined activities of alanine racemase and both D- and L-transaminase, as reported on *B. subtilis* and *B. anthracis*.

It was found that the glutamic acid racemase was not present in the enzyme preparation of *B. licheni*, while the very active alanine racemase was present. The formation of L-glutamic acid from the D-isomer by acetone dried cells of *Bacilli* other than *B. licheni* was neglesible. Therefore, there is little doubt that no glutamic acid racemase exists in genus *Bacillus* tested.

Studies on the Hardwood Dissolving Sulfate Pulp. Part III. Behavior of Hardwood during Prehydrolysis Sulfate Cooking (3) Factors on Removed Pentosan. (p. *857～863*)

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The factors which have influence on the removing of pentosan from hardwood in sulphate cooking with or without prceeding acid prehydrolysis were investigated. Sodium borohydride $NaBH_4$ as strong reducing agent, when incorporated to sulphate cooking liquor, was found to prevent pentosan from dissolving out of hardwood, both in normal sulphate cooking and prehydrolysis-sulphate cooking, as shown by the increased amount of remained pentosan in pulp. The same effect was also found in pretreated hardwood chip with neutral or weak-alkaline aqueous $NaBH_4$ solution followed by normal sulphate cooking. These phenomena should not be explained simply be chemical stabilization of pentosan itself without considering the effects derived from the change of cellulose fine structure which would be of great importance to restrain pentosan from dissolving out.

The adsorption of pentosan on cellulose fibers during sulphate cooking was proved to play a important roll also in sulphate cooking of prehydrolyzed chip with respect to the amount of remained pentosan in resulted pulp, thus the pentosan in resulted pulp was distinctly reduced by dividing the sulphate cooking into two stages and renewing the first liquor with dissolved pentosan by the second fresh liquor, even when the same amount of total active alkali was used as in normal one-stage sulphate cooking.

Studies on Glutamic Acid Fermentation. Part I. A Glutamic Acid-Producing Bacterium. (p. *863~866*)

By Shinji DOI, Yasuyuki KANEKO, Yasuhiro HASEGAWA, Isamu ADACHI and Toshihisa FURUKAWA

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The authors isolated a glutamic acid-producing bacterium from Okazaki, Aichiken, seqage. In 10% glucose medium, more than 40 mg of free L-glutamic acid were synthsized per ml of medium in about 4 days in shaken culture.

Cultures of this organism on nutrient agar: Gram-positive, non-sporeforming, non-acid-fast, and non-motile rods with rounded ends ($0.8\sim1.0\times1.1\sim2.8\,\mu$), granulations demonstrable with methylene blue stain; characteristic angular arrangements similar to Corynebacterium. Colonies are smooth, round (3~4 mm), entire, slightly elevated, dull, and milky white. No odor.

Acid but no gas is produced from glucose, fructose, mannose, sucrose and salicin; and maltose, lactose, trehalose, melibiose, raffinose, inulin, glycogen, starch, mannitol, and sorbitol are not fermented. Acid production is weak with principally L-(+)-lactic acid from glucose in static condition. Catalase, urease and lipase are positive; galatin and casein are not liquefied; indole, hydrogen sulfide and ammonia are not formed; nitrate is actively reduced to nitrite; acetylmethylcarbinol production is weak; and litmus milk is unchanged. The cells resist heating to 55°C, for 3 but not for 15 minutes in skim milk.

The above data indicate that the new isolate is closely related to the genus Microbacterium in the family Corynebacteriaceae, and resembles *Microbacterium flavum* in respect that the two strains do not hydrolyze starch and produce no acid from maltose, but the former differs from the latter in pigmentation, lipolytic activity, acid production from salicin, heat resistance, and direct fermentative production of high yield of free L-glutamic acid from sugars. Therefore, the authors conclude that this organism is a new species belonging to the genus Microbacterium, and propose to give to name, *Microbacterium salicinovorum* to this strain, in relation to the fermentation of salicin.

Biochemical Studies on Rice Plant. Part I. On the Nucleic Acid in Rice Plant Leaves. (p. *867~873*)

By Hitoshi OTSUKA*, Kinziro TAMARI**, Nagahiro OGASAWARA**

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The mixed nucleic acids were extracted from seven species of rice plant leaves by 10% (W/V) NaCl at 80°C. The ratio of RNA/DNA was 7.48~132.12.

The free ribonucleic acids which were isolated from mixed nucleic acids, showed typical nucleic acid absorption spectra.

When the free nucleic acids were hydrolyzed with 1 N- HCl, only the bases adenine, guanine, cytidylic acid, and uridylic acid cold be detected by paper chromatography. And quantitative analysis for purines and pyrimidines of the ribonucleic acid fraction was made.

The ratio of the purines and pyrimidines in ribonucleic acid was 0.93~1.345.

Decomposition of Glycol Chitin by Exocellular Cnitinase of the Black-koji Molds. (p. *873~878*)

By Satoshi OKIMASU and Akira ŌTAKARA

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The presence of exocellular chitinase in several black-koji molds has been investigated using glycol chitin, a water soluble derivative of chitin, as the substrate.

The action of crude enzyme upon the glycol chitin solution brought about the rapid reduction of viscosity and the production of reducing sugars, accompanied with the positive Morgan-Elson reaction. Crude enzyme also decomposed the insoluble substrate like regenerated chitin as well as glycol chitin.

Being determined chitinase activity using this substrate, it was found that one of the tested molds was most active in the production of exocellular chitinase. The optimum pH showed 3.6~4.0 and the optimum temperature was about 50°C.

From the experimental results obtained, it was concluded that glycol chitin was more suitable than such other substrates as regenerated chitin, chitodextrin, carboxymethyl chitin and chitosan used by earlier workers, because it was hardly depolymerized and deacetylated in the course of the preparation and its solution was very stable.

Studies on Egg Yolk Proteins. Part II. Protein Composition of Yolk Granules and Yolk Plasma. (p. *879~882*)

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Undiluted hen's egg yolk (I) was separated into yolk plasma (II) and yolk granules (III) by centrifugation at 100,000×g for 3 hours. Of the total P of I, 69% was present in II and 31% in III. About 96% of P of II was lipid P and the only 2% was phosphoprotein P. On the contrary, 69% of P of III was phosphoprotein P. At least 94% of the phosphoprotein P of I was present in III. The protein compositions of I, II, and III were investigated electrophoretically. The major portion of the protein of II was β-lipovitellin, which accounted for 70% of the total proteins of II. The main components of the protein of III were α-lipovitellin and phosvitin, which accounted for 62% and 26%, respectively, of the total proteins of III. All of the phosvitin was present in III. It was found that 21%, 46%, 9% and 24% of the total egg yolk proteins were α-lipovitellin, β-lipovitellin, phosvitin and livetin, respectively.

Studies on Egg Yolk Proteins. Part III. Isolation of Phosvitin from Yolk Granules. (p. *882~885*)

By Kiyozo HASEGAWA

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Yolk granules were used as a starting material for isolation of phosvitin. The purification steps were followed by electrophoretic analysis. To remove the free lipid, the yolk granules were dissolved in 5% NaCl solution and centrifugated at 100,000×g for 5 hours. The lower protein solution (fraction 2a–1) was collected careful siphoning. The fraction 2a–1 was diluted 4-fold with water and centrifuged. Almost all of the phosvitin remained in the supernantant (fraction 2b–23) The fraction 2b–23 was made 0.09 M with regard to $MgSO_4$ and, after centrifugation, the precipitate of crude phosvitin (fraction 2b–23–1) was obtained. The contents of phosvitin in the fractions 2a–1, 2b–23 and 2b–23–1 were found to be 25%, 41%, and 80% of the total proteins of the respective of the fractions. Phosvitin was extracted from the fraction 2b–23–1 with 0.4 M ammonium sulfate at pH 4, and followed by dialysis against saturated ammonium sulfate solution, against 2 M NaCl solution and against water. Solids of phosvitin were prepared by lyophilization. A typical preparation contained 10.06% phosphorus and 11.1% nitrogen (molar N/P ratio = 2.46).

Colorimetric Determination of Mercury with Copper Diethyldithiocarbamate and Estimation of the Solubility of Some Organic Mercury Compounds. (p. *885~888*)

By Yoshio HAMAMOTO and Masato KOTAKEMORI

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Studies were carried out to determine mercury by colorimetry with the use of copper diethyldithiocarbamate. This compound was found to be satisfactory in selectivity as coloring agent, and was free from the interference of impurities. In application to determine mercury in the organic mercury compounds, there was no need to consider the stability of the developed color and, the solutions obtained by decomposing sample compounds by 6 N hydrochloric acid could be directly used in colorimetry with good accuracy and convenience.

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Studies on the Changes of Milk Proteins by *Penicillium roqueforti*. Part IV. Consumption of Free Amino Acids and Effects of NaCl, Anaerobic Culture, and X-ray Irradiation. (p. *889~892*)

By Tsuneaki IMAMURA

(*Fac. of Agr., Okayama Univ.*)

Amino acids liberated from milk proteins were consumed by *P. roqueforti* as C- or N-sources and the states of such consumption have not been investigated. In this experiments, the ralative consumption of various amino acids was studied under the conditions of aerobic, anaerobic (removed about 90% of O_2 from air) and addition of 4 or 9% of NaCl which were similar conditions of Roquefort cheese. Effects of X-ray irradiation at 4000 or 2000 r were also studied to obtain basic knowledges on the changes of ripening by irradiation. Consumption rate was determined by decrease of NH_2-N in medium with Van Slyke method. On the normal aerobic culture, alanine, valine, tyrosine, and aspartic acid were consumed at high levels and less consumable ones were leucine, arginine, phenylalanine, methionine, and histidine. Such relative consumption rates were almost not changed by anaerobic condition and addition of NaCl less than 9%, excepts of glycine and glutamic acid. Proteolytic activity of cell extract was decreased by addition of NaCl.

Studies on the Changes of Milk Proteins by *Penicillium roqueforti*. Part I. Breakdown of Amino Acids by Cell Extract. (p. *892~896*)

By Tsuneaki IMAMURA

(*Department of Agriculture, Okayama University*)

Manometric studies on the oxidation and decarboxylation of amino acids by mold's cell extract and considerations of their contribution to Roquefort cheese ripening has been done. Wahrburg manometer was used for determining the changes of O_2-uptake and CO_2 evolved and microdiffusion analysis for NH_3 evolved. Between the value of Qo_2 of 15 amino acids used, those of methionine, alanine, valine, threonine, and serine were much greater than the others in this order. The op-pH of amino acid oxidative activity was at 8.0 and its maximum activity was observed at comparatively earlier stage of incubation, i.e., 5th day. Decarboxylative activity was observed in only glutamic acid by estimating of CO_2 evolved and paper chromatographic identification of γ-amino butyric acid and its op-pH was at 5.0. Accordingly, the presence of other amino acid decarboxylase was caused by other microorganisms in Roquefort cheese. From these results, such amino acid decarboxylation will probably take place in the interior of cheese. The amino acid oxidative activity was slightly lowered by more than 50000 r X-ray irradiation, but the ratio of NH_3 evolved and O_2 uptake weae always about 2 which indicate occurrence of oxidative deamination.

Studies on the Cellulose-decomposing Bacteria in the Rumen. Part VI. About the Effect of Various Kinds Feeds to the Mesophilic and Aerobic Cellulose-decomposing Bacteria in the Rumen. (p. *896~900*)

By Akira AKASHI

(*Department of Zootechemical Science, Faculty of Agriculture, Kyushu University*)

(1) The mesophilic and aerabic cellulose-decomposing bacteria were isolated from the rumen of goat fed by the bran for 2 months, from which none of the mesophilic and aerobic cellulose-decomposing bacteria were isolated. This result explains that these microbes are the resident flora in the rumen.

(2) According the changing the fodder, the isolated strains from the rumen change, but these are not always same with the strains from the fodder. The type of the strains from the same fodder are fixed, and this is reproductive even in changing the experimental animal (in sheep or goat) and the experimental date.

(3) Cellvibrio which is the no-color type is always isolated from the every fodder except the ensilage, the hay and the rice-bran. Cytophage hutchinsonii (the yellow-transparent color type) and Cytophage krzemieniewskae (the black and brown color type) are in many caces isolated, and *Pseudomonas rubeolifacience* and Cell-vibrio (the yellow type) and *Sporocytophaga myxococooides* and *Cytopha albogilva* (the brown type) and *Cytophaga rutea* (the red type) are often isolated and *Sporocytophaga congregata* var. maroonica (the maroonic type) are rarely isolated.

The Fine Structure of Amylopectin. Part I. Chain Length of Amylopectin. (p. *900~905*)

By Kazuji OHASHI

(Department of Agricultural Chemistry, Faculty of Agriculture, University of Gifu)

By the addition of isoamylalcohol to about 1.0% starch solution, amylopectin was fractionated from corn-, potato-, kidney bean-, sorghum- and waxy rice starches. The uptake of iodine of these amylopectin fractions was studied by iodine potentiometric titration, and iodine affinities for the samples were quoted. It was found that waxy rice- and potato starch give amylopectin of zero iodine affinity, but all amylopectin fractions of the other starches showed small but unmistakable iodine binding capacities. The length of chain units of the amylopectin was divided into three parts according to the iodine binding capacities such as long-, middle-, and short unit chains. The amounts of long- and middle unit chains were calculated from the amount of iodine bound by the amylopectin. Thus, the amylopectin fractions of corn-, sorghum- and kidney bean starches were found to contain 3～7% of long unit chain, but no iodine complex was precipitated by the addition of iodine and potassium iodide from these amylopectin fractions. By the same treatment, amylose fraction, which had been added to the amylopection fractions, was precipitated completely. Therefore, it may be assumed that the long- and middle unit chains contained in amylopectin fractions would be a part of the amylopectin molecule itself.

The Fine Structure of Amylopectin. Part II. A Specific Amylopectin. (p. *905～911*)

By Kazuji OHASHI

(Department of Agricultural Chemistry, Faculty of Agriculture, University of Gifu)

The fraction (C-fraction), which is precipitated by forming a complex with isoamylalcohol but not with butanol, was fractionated from corn-, kidney bean- and sorghum starches, respectively. The yields of C-fraction sorghum, 6%; from various starches were as follows: corn, 6%; kidney bean, 9%. The absorption peaks of iodine complexes of amylose, C-fraction and amylopectin were 640～660mμ (blue), 600～620mμ (blue) and 530～570mμ (red-violet), respectively. The iodine affinities of starchy materials were as follows; amylose, 18.7～20.0%; C-fraction, 2.4～4.4%; amylopectin 0～1.3%. Therefore, it may be said that the C-fractions contain the linear polymer of 12～22% of starchy materials. The results of intrinsic viscosity and reducing power indicated that C-fraction is a polymer as amylose and amylopectin are. By the method of periodate oxidation studies, it was shown that the average lengths of unit chain in amylose, C-fraction and amylopectin are 460～920, 27～33 and 19～27 glucose residues, respectively. The C-fraction and amylopectin retrograded more slowly than amylose. The iodine complex of amylose precipitated in the solution of iodine- iodide, but those of the C-fraction and amylopectin did not. Therefore, it may be deduced that the C-fraction is a specific amylopectin having sufficient amounts of long linear (12～22%) to permit precipitation with isoamylalcohol.

On the Fat-globule Membrane Materials of Cow's Milk. Part VII. Change of Whey Protein by Emulsification (I). (p. *912～914*)

By Susumu KOYAMA

(Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo)

Putting all previous papers dealing with fat-globule membrane materials together, it was conceivable to expect that the protein constituting the fat-globule membrane lipoprotein originated in whey protein. In the work described in this paper, in order to make this respect clear, artificial emulsion was prepared by emulsifying butter fat in whey, and the formation of lipoprotein was studied.

Pure butter fat containing egg lecithin was emulsified in acid casein whey by Manton-Gaulin homogenizer, and emulsion obtained was separated.

The presence of artificial lipoprotein in cream and skim milk was examined by paper electrophoretic technique, and the lipid-free proteinous residue of the lipoprotein was assayed for nitrogen content.

Result obtained showed that artificial lipoprotein was formed by emulsifying butter fat in whey, and hardly migrated on paper electrophoresis. The nitrogen content of proteinous residue was markedly lower than that of lactalbumin or lactoglobulin, and similar to that of fat-globule membrane protein.

On the Fat-globule Membrane Materials of Cow's Milk. Part VIII. Change of Whey Protein by Emulsification (II). (p. *915～918*)

(Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo)

In the previous paper, an artificial lipoprotein was prepared by emulsifying butter fat in acid casein whey, and its electrophoretic property was studied. At the same time, the nitrogen content of the lipid-free proteinous residue of the lipoprotein was also determined. In the work described in this paper, following previous investigation, another properties of the artificial lipoprotein were studied, and also the identification of the protein constituting the artificial lipoprotein was attempted.

Results obtained showed that the artificial lipoprotein was sedimented by ultracentrifugation (19000 g, 60 sec.), and the proteinous residue after treatment of the lipoprotein with the alcohol-ether mixture hardly dissolved on 0.08 M NaCl solution.

From both results obtained in this and previous paper, it was recognized that the artificial lipoprotein prepared by emulsifying butter fat in whey was similar to the fat-globule membrane lipoprotein.

Further, the other result from identification of the protein constituting the artificial lipoprotein showed that the greater part of the protein originated in lactoglobulin.

Putting all these results together, it is considered that the protein consituting the fat-globule membrane lipoprotein is mostly originated in lactoglobulin.

Studies on the Nutritive Value of Grass Proteins. Part VIII. The Effect of Drying Methods upon the Digestibility of Soy Bean Leaf Protein. (5) The Effect of Drying Method by Non-aeration at Low Temperature. (p. *919~925*)

By Makoto KANDATSU and Tadahiko YASUI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The changes in some constituents of Soy been leaf during non-aerated drying at low temperature were followed.

In the course of drying, as proteins and Nitrogen Free Extracts were considerably lost, so the decomposition of those constituents is assumed to have occured.

The apparent digestibility coefficients of proteins and Nitrogen Free Extracts by rabbits decreased by drying about 44 and 36 per cent respectively, but that of crude fiber was higher than that of the fresh leaf and the same as that of the leaf dried in the sun.

Also all of the soluble proteins decreased and the apparent digestibility coefficients of all kinds of proteins were lowered, especially that of 0.3% NaCl soluble indicated negative value.

From the result of this experiment, it is suggested that the decrease of apparent digestibilities of proteins may be caused by the same phenomenon discussed in the previous papers, and from the summeries of these serial experiments (Part VIII, A-E), it is suggested that the decrease of apparent digestibility of leaf protein during drying may be caused mainly by the following two occasions. The one of them is that the proteins condense with carbohydrates during drying and turn into indigestible forms, and the other is that metabolic fecal nitrogen excretion in the feces increases considerably.

Studies on the Metabolism of Azuki Seed Protein. Part III. Hydrolysis of One Protein Fraction (C_3^{ppt}) in Azuki Seed by Azuki Protease (1) (p. *926~930*)

By Shigeaki TAKAKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu Uuiversity*)

From the point of view that the activity of azuki protease to the protein fraction C_3^{ppt} in azuki seeds varies with denaturing condition of the substrate, the investigations were carried out and the following results were obtained.

1) Optimum pH of azuki protease was 4.3~4.5, when the enzyme was incubated to C_3^{ppt} as substrate which was dissolved in mixtures of urea in following buffers at temperature 33; 0.1 M citrate buffer, 0.2 M acetate buffer, and 0.1 M phosphate buffer.

It was similar to the results with haemoglobin and casein as substrate both denatured by urea.

2) By the investigation of enzymatic hydrolysis of C_3^{ppt} in various denaturing conditions, the results were as follows.

a) Native C_3^{ppt} which had been dissolved in acetate buffer above mentioned was more hydrolyzed by azuki protease than freezing dried C_3^{ppt}.

b) C_3^{ppt} denatured by 6 M urea was hydrolyzed as about five times as native one, and the C_3^{ppt} solution from which urea was completely eliminated by dialysis gave the same hydrolytic rate with native C_3^{ppt}.

c) According to estimation of hydrolytic rate of C_3^{ppt} denatured by various concentrations of urea, C_3^{ppt} denatured by 3 M to 6 M urea displayed the maximum hydrolytic rate but being denatured by the urea concentration over 6 M the hydrolytic rate was decreased.

d) Sodium lauryl sulfate, sodium salicylate, guanidine hydrochloride, acetamide, and urethan were used as denatuaing agents instead of urea. An increase of the hydrolytic rate of C_3^{ppt} treated with 3 M guanidine hydrochloride was similar to 3 M urea, but with 3 M acetamide thy hydrolytic rate din not increase.

As sodium lauryl sulfate and sodium salicylate formed the precipitate with protein, and ureathan gelatinized the protein, then the estimation was impossible.

e) Treating C_3^{ppt} at temperature 60 for 30 minutes, the protein displayed the hydrolytic rate similar to native C_3^{ppt}.

3) Azuki protease was not inhibited by urea of 3M

concentration but urea of the concentration over 3M inhibited its activity.

Studies on the Metabolism of Azuki Seed Protein. Part IV. Hydrolysis of One Protein Fraction (C_3^{ppt}) in Azuki Seed by Azuki Protease. (2) (p. *930～933*)

By Shigeaki TAKAKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

Light scattering intensities of the azuki protein fraction C_3^{ppt} denatured by urea and guanidine hydrochloride of various concentrations were estimated and a relation between the intensities (molecular weight) and the hydrolysis of the protein by azuki protease was investigated.

1) Molecular weight of native C_3^{ppt}, which was dissolved in acetate buffer of pH 4.2 and 0.1 ionic strength, was 485,000.

2) Molecular weight of C_3^{ppt} denatured by 6 M or 8 M urea was 42,500, but in the case of C_3^{ppt} denatured by 3 M or 4 M urea a dissociation curve was observed only in lower concentration of the protein (below 0.2×10^{-2} g/ml concentrations).

3) Guanidine hydrochloride also acted the same behavior to C_3^{ppt} as urea.

4) C_3^{ppt} from which urea was eliminated by dialysis after the protein had been denatured by 6 M urea, exhibited molecular weight of more than 5 millions, but the hydrolytic rate of this C_3^{ppt} solution was similar to that of the native C_3^{ppt}.

5) When the relation between molecular weight of C_3^{ppt} and its proteolytic rate by azuki protease was investigated, C_3^{ppt} denatured by 3 M urea made scarcely a change in the point of molecular weight, but the proteolytic rate became about 5 times of that of native C_3^{ppt}. As the increase of urea concentration, molecular weight became about 1/10 of native one, while the digestability was constant.

From the above facts, it would not be molecular weight but the alteration of the internal protein structure before the molecular weight depression, what influences largely to the digestability.

Mutant Associated Antagonism in *Micrococcus glutamicus*. Part I. On the Aliphatic Amino Acid Requiring Strain (p. *934～938*)

By Kiyoshi NAKAYAMA, Zenroku SATO and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyôwa Hakkô Kôgyô Co., Ltd.*)

During the study of amino acid accumulation by autotrophic mutants of a glutamic acid accumulating bacterium, *Micrococcus glutamicus*, valine accumulation by isoleucine-less mutant was found to be increased by addition of leucine or valine. Serine-less strain showed only a slight growth in usual media. From these facts, attempts were made to study the amino acid antagonisms associated to our mutants.

In this paper, antagonisms associated to aliphatic amino acid requiring strain were dealt. Competitive antagonisms were found in the following amino acid groups in the growth of the mutants requiring one or two amino acids in the following groups; serine-threonine, isoleucine-leucine-valine and lysine-arginine. Completitive inhibition of the growth of isoleucineless mutant on α-amino-*n*-butyrate by leucine or valine was also found.

Requirements of glutamic acid, threonine and cystine were spared by proline, isoleucine and methionine, respectively.

Mutant Associated Antagonism in *Micrococcus glutamicus*. Part II. On the Aromatic Amino Acid Requiring Strain. (p. *938～942*)

By Kiyoshi NAKAYAMA, Zenroku SATO and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Hakko Kôgyô Co., Ltd.*)

Continuing the previous studies, mutant associated antagonisms in *Micrococcus glatamicus* were investigated.

The growth phenylalanine-less mutant on phenylalanine was inhibited competitively by tyrosine or tryptophane. The growth of tryptophane-less mutant was inhibited competitively by tyrosine or phenylalanine. But the growth of tyrosine-less mutant was inhibited only by phenylalanine and not by tryptophane. Cystine and crystein inhibited the growth of tryptophane-less mutant completely and the growth of phenylalanine-less mutant considably, while tyrosine-less mutant was not inhibited. Balances in concentration of tyrosine, phenylalanine and tryptophane were necessary for the growth of the mutants requiring these two or three amino acids.

Antagonism in histidine-less mutant was also studied.

On the Viability of the Sand Cultures of Acetone-Butanol Organisms Stored for a Long Period. (p. *942～944*)

By Bunzo ROKUSHO* and Yoshimi SAITO**

(**Faculty of Agriculture, Meiji University*)

(***Faculty of Engineering, Yamanashi University*)

The viability of sand cultures of 3 strains of *Clastridium* *acetobutylicum*, prepared by the method of Weizmann, which had been stored in laboratory for 13 to 38 years was tested. It was proved that the ability of producing solvents was not degraded even stored for 38 years in ordinary temperature, and produced 2100～2290 mg solvents per 100 ml of fermented corn or sweet potato mashes in less than 68 h.

The microscopic appearance of spores of these sand cultures was normal; but all spores in the fermented mashes (not in sand culture) stored for 29 years appeared seriously damaged and no sign of fermentation was recognized.

Biochemical Studies on the Blast Disease of Rice Plants. Par XIV. On the Ingredients of Rice Plants, Possessing the Stimulating Effects on the Germination and the Growth of Blast Mould Spores. (2) (p. *945～949*)

By Kinjiro TAMARI, Jun KAJI, Nagahiro OGASAWARA and Masamichi SUGA

(*Faculty of Agriculture, Niigata University*)

In the previous papar (Part XI) the authors reported on a crystalline volatile substance "Oryzarol", possessing a stimulating effect on the germination of blast mould spore, isolated from the steam distillate of young rice plants. In this communication it has been investigated on the steam distillate of rice straws, referring to the stimulating substance of the blast mould spore's germination.

A very small quantity of Oryzarol was found in the distillate and eugenol, iso-eugenol and palmitic acid were isolated as the principal components from the acidic fraction, the main fraction of the distillate. Eugenol and palmitic acid were proved to stimulate the germination of the blast mould spore, the former being more effective than the latter. Iso-eugenol was none stimulative. Eugenol and iso-eugenol were also found in the steam distillate of young rice plants.

Biochemical Studies on the Blast Disease of Rice Plants. Part XV. On the Ingredients of Rice Plants, Possessing the Stimulating Effects on the Germination and the Growth of Blast Mould Spores. (3) (p. *949～953*)

By Kenjiro TAMARI, Jun KAJI, Nagahiro OGASAWARA and Masamichi SUGA

(*Faculty of Agriculture, Niigata University*)

In the previous paper (Part XI) the authors reported on a crystalline volatile substance "Oryzarol", possessing a stimulating effect on the germination of blast mould spores, isolated from the steam distillate of young rice plants.

The present communication deals with the chemical structure of Oryzarol. The authors confirmed the chemical structure of Oryzarol to be identical with Eugenyl-palmitate.

Études sur la Fermentation Acétono-Butylixne. IV. Rôle de l'Acétate dans la Nutrition de Clostridium, Souche No 314 (p. *953～957*)

par Shinji DOI et Seinosuke SUGAMA

(*Department de Agriculturale Chemie, Faculté de l'Agronome, l'Université de Nagoya*)

1) Clostridium, souche acétone-butylique No. 314 exige le glutamate comme la seule source d'azote. La dose exigée de ce dernier s'augmente en présence des acides aminés concourant avec le glutamate.

2) En milieux dont le glutamate devient un facteur limitatif, les acides acétique et butyrique sont accumulés et l'acétone se diminue et la fermentation se change en acide.

3) L'exigence du glutamate est complêtement remplacée par l'acétate d'ammoniaque à la presque même concentration d'azote, mais elle n'est pas remplacée par le $(NH_4)_2SO_4$ ou par le NH_4Cl.

4) Le milieu minimum pour la croissance et pour la fermentation normale de la souche No. 314 a la composition suivante: glucose (20 g/l), K_2HPO_4 (500 mg/l), KH_2PO_4 (500 mg/l), $MgSO_4\cdot 7H_2O$ (200 mg/l), $FeSO_4\cdot 7H_2O$ (10mg/l), $MnSO_4\cdot 4H_2O$ (10mg/l), NaCl (10mg/l), hydrochlorure de thiamine (1 mg/l), acide para-aminobenzoïque (1 mg/l), biotine ($10\,\gamma$/l), L-glutamate (200 mgN/l) ou acétate d'ammoniaque (200 mg/l).

Studies on the Production of D-Araboascorbic Acid by *Penicillium*. Part II. Studies on the producers. (p. *958～960*)

By Takeshi TAKAHASHI, Moritaka MITSUMOTO and Hisashi KAYAMORI

(*Deparment of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology*)

The isolated Penicillium producing D-araboascrbic acid was classificated. According to the "Studies on the classification of the Penicillia" by Dr. Shigeo Abe, this strain belongs to *Penicillium notatum*.

The Penicillia, 97 species and 127 strains, type cultures in The Institute of Applied Microbiology, The University of Tokyo, are tested for the production of D-araboascorbic acid. It was made clear by paperchromatography and infra-red absorption spectra of osazones that the following strains produce D-araboascorbic acid. They are *P. decumbens*, *P. chrysogenum*, *P. chrysogenum* mut. *fulvescens*, *P. notatum*, *P. meleagrinum*, and *P. cyaneofulvum*.

Studies on Gluconic Acid Fermentation. Part I. Decomposition of Gluconic Acid by *A. niger*.

(p. *961∼965*)

By Yoshio ICHIKAWA and Hiroshi IMANAKA

(*The Research Laboratory of Tokyo, Fujisawa Pharmaceutical Co., Ltd.*)

Gluconate is decomposed by the cell suspension of *A. niger* and gives reducing materials. Adenosine triphosphate and magnesium ion faver the reaction and phosphorylated intermediates are formed. Using ion exchange resins, it was revealed that the principal parts of reducing meterials are acidic substances. By the fractionation of barium salts of reducing materials using Dowex 50, the main fraction was isolated as white powder. From the absorption curves of some derivatives and the results of paper chromatography, this substance is suggested to be 2-keto-3-deoxy-6-phosphogluconic acid (6-P-KDGA), the dehydration product of 6-phosphogluconic acid. When the period of gluconic acid fermentation is prolonged, 6-P-KDGA often accumulates in the fermented liquor, resulting in the reformation of reducing power after glucose was completely consumed.

Studies on Gluconic Acid Fermentation. Part II. Decomposition of Gluconic Acid by *A. niger*.

(p. *966∼971*)

By Yoshio ICHIKAWA and Hiroshi IMANAKA

(*The Research Laboratory of Tokyo, Fujisawa Pharmaceutical Co., Ltd.*)

2-keto-3-deoxygluconate (KDGA) is easily decomposed as well as gluconate by the cells of *A. niger* adapted to gluconate. Doudoroff showed that KDGA was decomposed to pyruvate and triose phosphate by *Ps. saccharophila*, but in this case, 6-phospho-KDGA liberates equimolar carbon dioxide and changes into deoxyribose-6-phosphate. Besides deoxyridose-5-phosphate, sedoheptulose-7-phosphate, glucose-6-phosphate, fructose-6-phosphate, and ribose-5-phosphate were detected as decomposed products. Deoxyribose is turnded into ribose-5-phosphate and gives the same products as the case of KDGA under the same experimental conditions. As it is recognized that gluconate is esterified to 6-phosphogluconate before dehydration, we suggest that the pathway of gluconate decomposition is as follows:

Gluconate→6-phosphogluconate→6-phospho KDGA→deoxyribose-5-phosphate → ribose-5-phosphate → pentose phosphate cycle.

Pyruvate and α-ketoglutarate are also found in the broth of gluconic acid fermentation and in KDGA decomposed liquor. As *A. niger* shows the activity of oxidation to these substances, TCA cycle seemes to operate as the terminal oxidation system.

Studies on the Synthesis of L-Amino Acids. Part II. A Synthesis of L-Proline from L-Glutamic Acid. (2)

(p. *972∼975*)

By Masao TANAKA, Teruo KISHI and Shukuo KINOSHITA

(*Tokyo Research Lab., Kyowa Hakkô Kôgyo Co., Ltd.*)

The mechanism of the previously reported formation of L-proline from γ-ester of L-glutamic acid by reduction with sodium borohydride was studied. The results are as follows:

1) As the formation of proline from the ester of glutamic acid was presumed to be a reaction (i) through 2-pyrrolidone-5-carboxylic acid, (ii) through glutamic acid γ-semialdehyde and Δ′-pyrroline-5-carboxylic acid, or (iii) through 2-amino-δ-hydroxyvaleric acid, experiments were carried out on these three reactions.

2) A finding that 2-pyrrolidone-5-carboxylic acid was not reduced with sodium borohydride is considered to obviate the possibility of the course (i).

3) The course (iii) was excluded, because α-amino-δ-hydroxyvaleric acid gave proline under the heating with hydrochloric acid, but did not give proline under the condition of the reduction of the ester of glutamic acid.

4) The reaction cource (ii) was considered to be the most probable, because of well known findings that the ready ring-closure of glutamic acid γ-semialdehyde gives Δ′-pyrroline-5-carboxylic acid and the catalytic reduction of the latter yields proline. Thus, Δ′-pyrroline-5-carboxylic acid was synthesized through glutamic acid-γ-semialdehyde and observed to be reduced to proline with sodium borohydride.

The mechanism of the formation of proline from γ-ester of glutamic acid by the reduction with sodium borohydride was concluded as follows: the reduction of the ester gave glutamic acid γ-semialdehyde, then its cyclized product, Δ′-pyrroline-5-carboxylic acid was reduced to proline.

It is interesting that this reaction mechanism is similar to that of biochemical formation of L-proline, and the reaction may be employed for the synthesis of many nitrogenous heterocyclic compounds.

Tritium Counting with a Windowless Gas Flow Counter.

(p. *976∼977*)

By Shigeki MIZUNO, Hajime TAKAHASHI and

Bunji MARUO

(*Division of Enzymology, the Insitute of Applied Microbiology, University of Tokyo*)

The method was described to determine the radioactivity of ^{3}H-compounds at infinite thickness by using a filter paper-disk. Tritiated sample was put on the filter paper-disk (1.9 cm diameter), placed in the centre of an iron planchet (5 cm^2) and was counted with a windowless gas flow counter after drying under an infrared lamp. Using this paper-disk method good proportionality between the total radioactivity of added ^{3}H-Adenosine ($0 \sim 1.0$ mg/cm^2, < 0.3 ml/disk) and the actual count was demonstrated.

Counting error was within ±10%. Counting efficiency was about 0.3% of the total disintegration events.

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